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Glucose is a key substrate for lactating and neoplastic mammary epithelial cells. Our purpose is to understand how lactating breast meets its need for glucose transport into Golgi, the site of lactose synthesis, and whether this mechanism has physiological, pathophysiological, or potentially therapeutic relevance in breast cancer. The scope of the research includes established cell lines, primary cells, and mammary glands, from both mice and humans. Major findings are: 1) in mouse and human mammary epithelial cells in culture and also in mouse mammary gland, the hormonal milieu of lactation induces GLUT1 and causes it to be targeted to Golgi, 2) GLUT1 targeting to Golgi is dynamic, rapidly reversible, and requires endocytosis, and 3) GLUT1 is virtually absent from the plasma membrane in MCF7 and MDA231 cancer cells even in the absence of lactogenic hormones, and is targeted to a non-Golgi compartment. The results suggest that GLUT1 is not responsible for plasma membrane glucose transport activity in breast cancer. A unique mechanism, possibly with effects on targeting and activity of other proteins, must underlie the sequestration of GLUT1 in breast cancer cells. These cells may express a novel glucose transporter, potentially an attractive therapeutic target.			
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Introduction

As of July 1, 1997, I became an Assistant Professor of Pediatrics at the Baylor College of Medicine in Houston Texas. I am affiliated with the Section of Neonatology and with Texas Children's Hospital and my lab is in the U.S.D.A./A.R.S. Children's Nutrition Research Center. Note that, since DOD policy did not allow the grant to be transferred from Washington University to Baylor College of Medicine, F. Sessions Cole, M.D. graciously agreed to serve as Principal Investigator and to subcontract the work to me at Baylor College of Medicine. Therefore, I am technically submitting this report on his behalf.

Glucose is critical to mammary epithelial cells because it serves as a fuel and as a building block for glycoproteins and glycolipids. Furthermore, it is the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production. Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein α -lactalbumin. Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescent state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young. Breast cancer cells also exhibit an increased demand for glucose. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal. The specific hypotheses to be tested are: 1) glucose transport into mammary epithelial cells is subject to a high degree of regulation, and 2) abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth. In order to test these hypotheses, the following specific aims were chosen: 1) description of the developmental and hormonal regulation of glucose transport in mammary gland, 2) identification of novel proteins involved in glucose transport in lactating mammary epithelia, and 3) examination of a possible association between abnormal glucose transport and the neoplastic phenotype. The scope of the work includes primary mammary epithelial cells, established mammary epithelial cell lines in culture, and intact mammary glands from mice and humans.

BodyResults and Discussion of Research Accomplishments to Date*Task 1: Description of the developmental and hormonal regulation of glucose transport and lactose biosynthesis in CIT3 cells and in mammary gland*

Previous Annual Reports have described the developmental and hormonal regulation of glucose transport in mouse mammary epithelial cells in culture and in mouse mammary gland. During the past year one manuscript based on this work has been published, and a second has been accepted for publication. These manuscripts are Appendices 2 and 3.

Thus, task one has been accomplished regarding the mouse. A model involving the induction of GLUT1 and its retargeting from the plasma membrane to the Golgi under the influence of the hormonal milieu of lactation is confirmed by the data. This model serves well for further experiments to understand the mechanism of the retargeting of GLUT1.

During the past two years, we have turned our attention to the human mammary epithelial cell, intending to accomplish the same understanding in cells in culture. It is not possible to carry out studies of the lactating human mammary gland in a parallel fashion. However, mammary epithelial cells can be isolated from milk during the first week of lactation, and from surgical specimens. We carried out our studies in commercially available cells (Clonetics, Walkersville, MD) derived from normal surgical specimens. This work is described in a manuscript which is in final draft form for submission, Appendix 4. An excerpt reads:

Immunofluorescent microscopy of human mammary epithelial cells in maintenance medium using anti-GLUT1 antibody demonstrated primarily plasma membrane distribution of GLUT1, as well as some intracellular staining, mostly in a perinuclear pattern. After exposure to prolactin-rich medium for 4 days, GLUT1 was specifically targeted intracellularly, demonstrating a perinuclear punctate pattern as well as apparent nuclear membrane staining. Plasma membrane staining was markedly reduced. In cells exposed to prolactin, perinuclear GLUT1 colocalized with ECFP-Golgi, a cyan fluorescent protein fused to a membrane-anchoring signal specific to (1,4-galactosyltransferase, identifying the medial/trans region of the Golgi. Immunocytochemistry of cells exposed to prolactin also demonstrated colocalization of GLUT1 with (α -lactalbumin, the milk whey protein that associates with galactosyltransferase to form lactose synthetase, and with (α -mannosidase II, a medial-Golgi marker. GLUT1 also showed partial colocalization with (β -COP, a cis-Golgi marker that also marks the trans-Golgi network and the ER-Golgi boundary. GLUT1 did not colocalize with BODIPY-TR ceramide, a trans-Golgi marker. A brief exposure to transferrin-Texas Red served to mark endosomes, while prolonged exposure marked lysosomes. Some perinuclear punctate staining of GLUT1 corresponded to endosomes, but not to lysosomes. Nonspecific signal was negligible under all conditions.

Upon exposure of human mammary epithelial cells to prolactin, GLUT1 is targeted primarily to the medial-Golgi, colocalizing with the components of lactose synthetase complex. The initiation of (α -lactalbumin synthesis that occurs at parturition is required for the initiation of copious milk production, but is neither the only factor nor the limiting factor controlling lactose synthesis. Our findings support the importance of GLUT1 for transport of glucose into Golgi, and suggest how a substrate required for lactose synthesis is delivered. Apparent nuclear membrane staining for GLUT1 seen in human mammary epithelial cells has not been reported previously in any cell type, and its significance is a matter for speculation and further study. The suggestion that GLUT1 does not solely act at the plasma membrane, but may function in an intracellular organelle as well, conceptually complements the well-known insulin-regulated targeting of GLUT4, and to a lesser extent of GLUT1, to their site of action, the plasma membrane, in fat and muscle cells. Our results indicate the existence of a prolactin-induced, cell type-specific, developmental stage-specific sorting machinery for GLUT1 in mammary epithelial cells. The identification of GLUT1 in endosomes suggest that GLUT1 sorting is a continuous, dynamic process. Further work delineating the molecular mechanism of GLUT1 sorting and the targeting determinants it recognizes should improve our understanding of a key regulatory step of milk production in the nursing mother.

We have also developed a ribonuclease protection assay for detection of all glucose transporters simultaneously. The principle for this is that a unique region of each glucose transporter mRNA, of between 150 and 400 nucleotides, differing in size by at least 20 bases, have been selected. PCR primers for each sequence have been made and used to synthesize PCR products with a T7 promoter at the 3'end of the antisense sequence. These PCR products served as substrate for T7 RNA polymerase, which made antisense RNA species for each sequence. Specifically, the sizes of the probes are: GLUT1, 187; GLUT2, 273; GLUT3, 234; GLUT4, 149; and GLUT5, 344. These were used as riboprobes after labeling with psoralen-biotin and used in the ribonuclease protection assay. If the corresponding mRNA is present, the labeled probe is detected, and a signal of the appropriate size is seen. If not, not. The strength of the signal is proportional to the amount of glucose transporter RNA present. Since each transporter gives a signal at a unique size, we are able to determine the amount of each transporter simultaneously. Figure 1 shows the results of a ribonuclease protection assay using probes for GLUT1-5 and for α -lactalbumin, β -casein, and galactosyltransferase, key indicators of differentiated function and lactose synthesis, in mouse and human mammary epithelial cells. Mouse cells show an induction of GLUT1 and galactosyltransferase on exposure to prolactin and hydrocortisone, but do not express α -lactalbumin or β -casein. Interestingly and surprisingly, in contrast to reports from others, this sensitive assay detects not only GLUT1 as the predominant glucose transporter, but also substantial GLUT4 and GLUT3, and a lesser amount of GLUT2.

Thus, I conclude that Task 1 has been essentially accomplished.

Task 2. Identification of novel proteins involved in glucose transport in lactating mammary epithelia

As reported in the 1999 Annual Report, we had concentrated on differential display as the method of choice for pursuit of this task. One gene previously identified, LDH-A, has been further studied, and evidence regarding its induction by lactogenic hormones was provided in previous reports. We also showed a 2-3 fold induction of LDH activity in last year's report. In order to determine whether the presumed induction of LDH-A mRNA by prolactin and hydrocortisone was also associated with an increase in LDH-A protein, LDH isoform analysis was conducted. The results demonstrated no significant change in LDH-A protein, and led to a new interpretation of the positive differential display result and the apparent difference in enzyme activity. The results suggest that the specific activity of LDH may be increased by lactogenic hormones, since the amount of protein did not change. While interesting, this observation is not relevant to our specific aims, and we have not pursued LDH-A induction further.

Previous Annual Reports included progress with several novel expressed sequence tags identified by this process. These ESTs correspond to genes expressed in response to lactogenic hormones but are actually unlikely to be specifically related to regulation of glucose transport. I periodically search the rapidly accumulating gene databases for homologies to these sequences. As reported in 1999, one of the novel sequences we identified showed an extremely high degree of homology with the newly deposited sequence of human sec24, a protein required for vesicular transport from endoplasmic reticulum to Golgi. Our sequence appears to be mouse sec24. It is not surprising that a protein required for vesicular transport along the secretory pathway would be induced during lactation, and this is presumably a true positive, but irrelevant to our current specific questions regarding glucose transport. Nevertheless, it does point out an interesting area for future investigation.

As I pointed out in the 1999 Annual Report, because a number of recent papers have appeared pointing out the difficulties and large amount of efforts and resources required to bring differential display analyses to a conclusion, and because of rapid progress of the human genome project information which will facilitate identification of the current sequences, and because of rapid development of DNA microchip arrays, which offer many advantages over differential display, I deferred further pursuit of the ESTs at that time. I do continue to search the databases routinely for homology to the currently identified sequences.

As reported in 1999, because of a report of GLUT1-C-terminal binding proteins that associate with GLUT1 and alter its targeting (Bunn et al., 1999), we undertook PCR-based screening to attempt to identify a GLUT1-CBP related protein in lactating mouse mammary gland. Four sets of primers were chosen from the coding region, and all possible permutations were used with cDNA from a lactating mouse mammary gland cDNA library. No PCR products were obtained, suggesting that neither GLUT1CBP nor any other related protein is expressed, or that all areas chosen diverge sufficiently in structure such that no PCR product was obtained. I will follow the literature in this area closely and continue to screen for other GLUT1CBPs, if any others are identified.

I described in the 1999 Annual Report a fascinating and potentially very direct

approach involving the use of dodecyl maltoside and Blue Native protein electrophoresis. This approach is designed to not disrupt associations between proteins; thus, the signal on the gel reflects the sum of the molecular weight of the protein being detected and the molecular weight of other protein(s) associated with it. When this procedure is carried out on iodixanol density gradient purified Golgi fractions from lactating mammary gland or from CIT3 cells grown in secretion medium (data not shown), a single broad band running at about 130 kD was identified. The simplest interpretation of this would be that GLUT1 is associated with a single protein of about 80-90 kD. This would presumably be a Golgi resident protein responsible for retaining GLUT1 in the Golgi. Note that virtually no signal is identified at about 45 kD, where monomeric GLUT1 would be seen; thus, under these conditions, virtually no Golgi GLUT1 exists as a free integral membrane protein. The next steps I described were to further purify this protein by denaturing gel electrophoresis and by 2-D gel electrophoresis, to obtain peptide sequence information, and to identify the associated protein as a known protein or to clone it. The most efficient purification scheme would use GLUT1 antibody to immunoaffinity purify GLUT1 and its associated proteins. This requires a large amount of antibody, and necessitated that we raise our own antibody to GLUT1 rather than rely on kind gifts. Rabbit antibody was raised against the sequence SGFRQGGASQSDKT, amino acids 464-477, and also to PEELFHPLGADSQV, amino acids 478-491. This antibody has been purified and characterized. To improve our purification scheme, we have recently needed to concentrate on preparation of a more highly enriched and intact Golgi fraction, and have succeeded using nitrogen bomb rather than homogenization (data not shown). Thus, we have developed an approach to identification of the GLUT1-associated protein. This will be the goal of a subsequent research proposal.

Task 3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype

I chose to begin our studies of neoplastic cells with well established cultured cell lines, so we could develop an understanding of the mechanisms of glucose transport in neoplastic mammary epithelia before utilizing human tissue. These experiments were generally designed to test the hypothesis that neoplastic mammary epithelial cells would demonstrate high levels of glucose transport activity and GLUT1 protein to support a high rate of glucose utilization, and that GLUT1 in these cells would be targeted primarily to the plasma membrane, again in order to support a high rate of glucose utilization. The hope I set forth in my proposal was that activation of the Golgi targeting mechanism for GLUT1 in a neoplastic cell might serve to deprive the cell of substrate, limit its growth, and make it more vulnerable to chemotherapy.

In the 1998 Annual Report I described our results with MCF7 cells, a non-metastatic line, and MDA231 cells, a metastatic line. In brief, MCF7 cells showed very low glucose uptake and were not responsive to prolactin and hydrocortisone. In contrast, MDA231 appeared to demonstrate an exaggeration of the pattern seen in CIT3 cells, with a very high glucose uptake under basal condition, and 73% inhibition by prolactin and hydrocortisone, consistent with very high total cellular levels of GLUT1 with highly regulated changes in subcellular distribution paralleling those seen in CIT3 cells.

However, hormonal treatment affected neither the amount nor the subcellular targeting of GLUT1 in MDA231 cells, and there was very little GLUT1 detected in the homogenate and plasma membrane fractions, suggesting that GLUT1 was not the glucose transporter responsible for the very high rates of glucose transport activity.

We have concentrated during the past two years on understanding the intracellular compartment to which GLUT1 is targeted and the mechanisms involved. This has proven to be a difficult task; note that Appendix 2, an in press manuscript describing GLUT1 targeting in normal mouse mammary epithelial cells, includes extensive immunocytochemical analysis to characterize this compartment, which can be distinguished from classical Golgi stacks but is nevertheless highly sensitive to Brefeldin A and very low in density. It was necessary to fully understand glucose transporter targeting in normal cells as a basis for comparison for neoplastic cells.

These studies have involved two major areas, immunocytochemistry in fixed cells, and immunofluorescence due to the expression of GLUT1-EGFP fusion proteins in living cells. The use of fluorescent fusion proteins of GLUT1 is attractive because their use avoids potential artifacts of fixation, allows the study of the same cells over time, permits studies of exocytosis and endocytosis, not just steady state distributions, facilitates studies of organized cells in extracellular matrix, permits Fluorescence Resonance Energy Transfer (FRET) studies of molecular interactions, permits targeting of chimeric proteins to be evaluated in an antibody-independent fashion, and confirms we are studying GLUT1, not a novel, lactation-specific Golgi glucose transporter isoform that shares the GLUT1 epitope. In the 1998 Annual Report, the successful construction of an expression vector for a GLUT1-EBFP (enhanced blue fluorescent protein) fusion protein, and the expression of this fusion protein by liposome-mediated transfection of CIT3 cells, was discussed. We initially chose blue fluorescent protein constructs so that green signal could be reserved for other proteins or markers. However, work during the past two years led us to conclude that the blue fluorescent protein constructs were plagued by rapid photobleaching and such a weak blue fluorescent signal that their use was limited to static images, and therefore not useful for studies of trafficking kinetics. We also explored the possible usefulness of red fluorescent proteins, but found artifactual targeting prohibited their use. This work is described in Appendix 5, Usefulness of Blue, Green, and Red Fluorescent Protein Chimeras in the Study of GLUT1 Glucose Transporter Intracellular Targeting in Mouse Mammary Epithelial Cells, a draft manuscript. The abstract reads:

GLUT1 is the only glucose transporter isoform expressed in the mammary gland. Hormonally regulated subcellular targeting of GLUT1 from the plasma membrane to Golgi is important for lactose synthesis in lactation. The purpose of this study was to generate mammary epithelial cells expressing blue, green or red fluorescent protein chimeras of GLUT1 in order to assess their usefulness in the study of GLUT1 intracellular targeting. GLUT1 cDNA was subcloned into pEBFP-N1, pEGFP-N1 and DsRed1-N1 (Clontech). EGFP and EBFP are enhanced green and blue variants of the well studies green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and DsRed1 represents a red fluorescent protein from the anemone relative *Discosoma striata*.

After liposome-mediated transfection of CIT3 mouse mammary epithelial cells, fluorescent signal was observed in transient transfections. GLUT1 redistribution from plasma membrane to Golgi in mammary epithelial cells upon conditions mimicking lactation was demonstrated using GLUT1-EGFP chimeras. As opposed to the easily detected green fluorescent signal of these chimeras, the blue fluorescent signal of GLUT1-EBFP fusion chimeras was very weak, and susceptible to rapid photobleaching, thus limiting their usefulness. The newly reported red fluorescent protein DsRed1 has an easily detected red signal, but did not exhibit the expected intracellular pattern seen with EGFP and EBFP. The GLUT1-DsRed1 chimera did not follow the distribution of native GLUT1, nor did it co-localize with GLUT1-EGFP chimera. Thus, chimeras including this red fluorescent protein were not useful in the study of GLUT1 trafficking. Our findings raise major concerns, whether DsRed1 will be useful in the study of other proteins in different cell types. Our future studies will utilize chimeras of GLUT1 and EGFP, rather than EBFP or DsRed1, for co-localization and intracellular trafficking.

Studies were carried out using both transient transfection, with analysis at 24-96 h, and by stable transfection, using 1) CaPO4, 2) DEAE-Dextran, 3) Polycationic transfection reagent (SuperFect), and 4) Liposome-mediated transfection (LipoFectAmine). These studies were done systematically to determine the optimal transfection system. For further details, please refer to Appendix 6, the draft manuscript "Cationic liposome-mediated stable transfection of CIT3 mouse mammary epithelial cells." The abstract reads:

Transfection of mammary epithelial cells can provide important insights into the molecular biology of milk secretion and breast cancer. Difficulties transfecting these cells have been reported previously. We compared the relative transfection efficiency of CaPO4, DEAE-Dextran, cationic polymers and cationic liposomes. CIT3 mouse mammary epithelial cells were transfected with green or blue fluorescent protein expression vectors. Transfection efficiency was highest for cationic liposomes compared to all other transfection methods ($p<0.05$). Fluorescence was detected in 31+4% of these stably transfected colonies. Cationic liposome-mediated stable transfection efficiently yields stably transfected mammary epithelial cells with a high frequency of exogenous gene expression.

We devoted our major recent effort to studies of expressed GLUT1-EGFP fusion proteins in normal mouse mammary epithelial cells. These studies are essential for interpretation of studies in cancer cells and are described in Appendix 7, a draft manuscript, "Dynamic Hormonally Regulated Targeting of the GLUT1 Glucose Transporter in Mouse Mammary Epithelial Cells." The key advance is the study of targeting in the same cell over time, permitting an understanding of the dynamics underlying the steady state as well as a model system for perturbation with specific agents that influence transporter targeting. The abstract reads:

The mammary gland is unique in its requirement for transport of free glucose into the Golgi, the site of lactose synthesis. GLUT1 is the only known isoform of glucose transporter expressed in the mammary gland. Lactogenic hormones cause targeting of

GLUT1 to the Golgi. Mechanisms of intracellular targeting of GLUT1 are not known. Hypothesis: GLUT1 intracellular targeting under hormonal stimulation is dynamic. Methods: To construct fusion proteins of GLUT1 and green fluorescent protein (GFP), cDNA was subcloned into pEGFP-C1 and pEGFP-N1 (Clontech). GFP-GLUT1 fusion proteins were expressed in CIT3 mouse mammary epithelial cells (M.C. Neville, University of Colorado) using liposome-mediated transfection. Cells were maintained in growth medium, or exposed to prolactin at different concentrations and for different times. To further define the intracellular trafficking mechanisms involved in GLUT1 targeting and recycling in mammary epithelial cells, cells were also exposed to inhibitors, which affect GLUT1 and GLUT4 targeting in muscle and fat cells, including bafilomycin A1, wortmannin and staurosporine. Cells were studied at 37°C. Time lapse fluorescent images were captured by an uncooled CCD camera. Results: In growth medium, the N- and the C-terminal fusions of GFP and GLUT1 demonstrated plasma membrane targeting. Prolactin changed subcellular targeting of both fusion proteins to an intracellular punctate pattern, as seen with native GLUT1. Time lapse images revealed dynamic trafficking of GFP-GLUT1 fusion proteins. Upon exposure to prolactin, GLUT1 fusion proteins were redistributed intracellularly, starting after approximately 50-60 minutes, with maximal intracellular targeting within 90-110 minutes. When the cells were returned to GM, most of the changes were reversible. Bafilomycin A1, which causes arrest of endosomal acidification, caused central coalescence of GFP-GLUT1 and the loss of peripheral vesicles. Wortmannin and staurosporine effects on internalization of GLUT1 were not specific to mammary epithelial cells or prolactin exposure, but supported basal recycling of GLUT1. Conclusions: Our results demonstrate a basal constitutive GLUT1 membrane-recycling pathway between an intracellular pool and the cell surface in mouse mammary epithelial cells, which targets most of the GLUT1 to the plasma membrane in maintenance medium. Upon exposure to prolactin GLUT1 is specifically targeted intracellularly. This change takes place within hours, and may support glucose transport as a rate-limiting step for lactose synthesis during lactation. Arrest of endosomal acidification by bafilomycin A1 disrupts this process, which implies trafficking via endosomal pathways.

As reported in the 1999 Annual Report, we have also studied cancer cells. We continue to concentrate on the MCF7 and MDA231 cells at this point. Studies using transient transfection are showed the unique intracellular targeting of GLUT1-EGFP to intermediate-size, GLUT1-dense vesicles in MCF7 cells in growth medium. Little colocalization with Bodipy-TR ceramide was seen. This is particularly interesting, since we were unable to detect the cell's own GLUT1 using our standard immunofluorescence techniques. Strikingly similar results were obtained in MDA231 cells. The cis-Golgi marker beta-COP showed no colocalization with the GLUT1-EGFP fusion protein in these cells.

We reported in 1999 a considerable investment of effort to isolate and expand stably transfected clones of cells expressing the fusion proteins, and we had several unique colonies for each cell type at that point. Multiple independent clones must be studied in order to conclude that results are not a function of clonal variation or of inappropriate levels of fusion protein expression. A very significant obstacle to our

progress was that expression of exogenous protein did not occur in the thawed cells. This appears to be due to toxicity of the GFP under prolonged exposure, and recent modifications in the structure of GFP by Stratagene appear to overcome this problem. This necessitates that we discard our frozen stocks and redo the work with the modified GFP vector. We continue to see many advantages to the use of a stable transfection system, with a greatly reduced potential for artifact and ease of expression of a second fluorescent protein.

We have also used the ribonuclease protection assay to study GLUT1-5 in MCF7 and MDA231 human breast cancer cells. The results show that GLUT1 and GLUT4 are the major transporters, and that GLUT2, GLUT3, and GLUT5 are not expressed (Figure 2). Also, mRNAs for the key proteins of lactation, α -lactalbumin, β -casein, and galactosyltransferase, are not detectable in these cells (Figure 3). Since GLUT1 and GLUT4 are intracellular in these cells, this suggests that it is a novel glucose transporter, or "oncotransporter," that is important in these cells.

We also sought under Task 3 to study effects of altered glucose transport on mammary epithelial cell phenotype, including transport properties and nutrient utilization. A key requirement of a model system is the ability to synthesize lactose, and we expended considerable effort trying to identify specific conditions to maximize this. Despite this, even the use of a radioisotopic method to detect very small amounts of lactose was unsuccessful. We used the ribonuclease protection assay to try to identify hormonal conditions that would support lactose synthesis. Figure 4 demonstrates that even with triple the usual concentrations of prolactin and hydrocortisone, there was no expression of α -lactalbumin in our cultured cells. This explains the lack of lactose synthesis. The absence of β -casein and the lack of increase in galactosyltransferase serve to confirm the lack of differentiated function even under these conditions. A critical future direction for research is the development of a valid *in vitro* system to study lactose synthesis.

In summary, with respect to Task 3, at this point we have demonstrated that the neoplastic phenotype of mammary epithelial cells, but not the normal phenotype, includes an absence of plasma membrane targeting of GLUT1, which is instead targeted to an intracellular compartment which does not appear to be Golgi. The results therefore indicate that the intracellular targeting of GLUT1 is not caused by an abnormally timed triggering of the GLUT1 targeting mechanism operative during lactation. Rather, it represents a mechanism specific to the neoplastic cell. We have defined the intracellular compartment in which GLUT1 resides in normal cells, with the realization that Golgi-related vesicles are an important destination. Documenting this, and the dynamic nature of glucose transporter targeting in these cells, consumed the time and resources available from this project. We intend in a future proposal to execute studies of human tumors and transplanted mouse mammary glands now that we have identified this compartment and useful markers for it. The results also suggest that neoplastic cancer cells do not rely on GLUT1 for transport of glucose across the plasma membrane, and suggest another focus for future research will be identification of the protein which does facilitate the transport of glucose across the plasma membrane of breast cancer cells.

The specific hypotheses to be tested in this project were: 1) glucose transport into mammary epithelial cells is subject to a high degree of regulation, and 2) abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth. The results demonstrate that glucose transport into mammary epithelial cells is indeed subject to a high degree of regulation. The results also clearly demonstrate that the neoplastic state is associated with abnormal glucose transporter targeting and unrecognized mechanisms for glucose transport across the plasma membrane. The results will serve as a solid foundation for my future work in this area.

Key Research Accomplishments

- In normal mouse and human mammary epithelial cells in culture, the GLUT1 glucose transporter is induced by prolactin and hydrocortisone and undergoes a change in intracellular trafficking from a plasma membrane distribution to an intracellular pattern consistent with targeting to the Golgi.
- In mouse mammary gland, the GLUT1 glucose transporter is induced during pregnancy and lactation and undergoes the same change in intracellular trafficking described above.
- Golgi targeting of GLUT1 in mouse mammary gland during lactation is reversible and is influenced by suckling and weaning.
- In MCF-7 and MDA-231 human cancer cells, GLUT1 is targeted not to the plasma membrane, but to an intracellular compartment, and is not subject to hormonal regulation.
- Liposome-mediated transfection can be used efficiently in both normal and neoplastic mammary epithelial cells.
- In cancer cells, GLUT1 levels and subcellular targeting are not correlated with glucose transport activity. The regulation of glucose transport activity by prolactin and hydrocortisone in these cells suggests the possibility of another glucose transporter playing an important role.
- Fusion proteins of GLUT1 with green fluorescent protein reveal dynamic intracellular trafficking of GLUT1 in both normal and neoplastic cells and will be an important tool in understanding aberrant glucose transporter targeting in cancer cells.
- An intact endosomal pathway is required for appropriate GLUT1 targeting.
- Ribonuclease protection assay demonstrates that normal mammary epithelial cells express GLUT1, GLUT2, GLUT3, GLUT4, and not GLUT5, but only GLUT1 and GLUT4mRNA is detected in neoplastic cells.

Reportable Outcomes

1. Manuscripts, Abstracts, Presentations

Manuscripts

1. Nemeth, BN, Tsang, ST, Geske, RS, Haney, PM. Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland, *Pediatric Research*, 2000; 47:444-450.
2. Haney, PM. Localization of the GLUT1 glucose transporter to Brefeldin A-sensitive vesicles of differentiated CIT3 mouse mammary epithelial cells, *Cell Biology International*, in press.
3. Riskin, A, Nannegari, V, and Haney, PM. Cationic liposome-mediated stable transfection of CIT3 mouse mammary epithelial cells, in preparation.
4. Riskin, A, Nannegari, V, and Haney, PM. Usefulness of blue, green, and red fluorescent protein chimeras in the study of GLUT1 glucose transporter targeting in mouse mammary epithelial cells, in preparation.
5. Riskin, A, Nannegari, V, and Haney, PM. Prolactin induces targeting of GLUT1 to Golgi in human mammary epithelial cells, in preparation.
6. Riskin, A, Nannegari, V, and Haney, PM. Dynamic hormonally regulated targeting of the GLUT1 glucose transporter in mouse mammary epithelial cells, in preparation.

Abstracts and Presentations

1. Haney PM. Structural determinants of glucose transporter targeting. Tenth Annual Mead Johnson Neonatal Nutrition Symposium, Marco Island, FL, December 1995.
2. Haney PM. Exposure of mammary epithelia to lactogenic hormones causes Golgi sequestration and plasma membrane polarization of glucose transport activity. *Ped Res* 1996; 39:89A (SPR platform presentation).
3. Nemeth BA, Haney PM. Preferential targeting of GLUT1 to Golgi of mouse mammary epithelial cells during lactation is rapidly but reversibly lost during premature weaning. *Ped Res* 1997; 41:70A (SPR poster symposium presentation).
4. Haney, PM. Regulation of glucose transport in quiescent and lactating mammary epithelia. Proceedings of the Department of Defense Breast Cancer Research Program Meeting 1997; vol. 1: 269-270.
5. Haney, PM. Premature weaning alters glucose transporter targeting in lactating mouse mammary gland. *Mol. Biol. Cell* 1997; 8, 307a (ASCB poster presentation).
6. Geske, S, Haney, PM. Developmental regulation of the amount and subcellular targeting of the GLUT1 glucose transporter in mouse mammary gland. *Ped Res* 1998; 43:75A (SPR poster symposium presentation).
7. Haney, PM. Hormonal regulation of the amount, activity, and subcellular targeting of GLUT1 in normal and neoplastic mammary epithelial cells. *Ped Res* 1998; 43:77A (SPR poster symposium presentation).
8. Riskin, A, Nannegari, V, Haney, PM. Efficient transfection of CIT3 mammary

epithelial cells using liposomes. FASEB J. 1999; 13:A432 (FASEB poster presentation).

- 9. Haney, PM. Lactogenic Hormones Cause Glycosylation-Independent Targeting of the GLUT1 Glucose Transporter of Mammary Epithelial Cells to a Subcompartment of *cis*- and/or medial-Golgi. Ped Res 1999; 45:90A (SPR poster symposium presentation).
- 10. Riskin, A, Nannegari, VH, Haney, PM. Prolactin induced GLUT1 targeting to medial-Golgi in human mammary epithelial cells. FASEB J. 2000; 14:A101. (FASEB poster presentation).
- 11. Riskin, A, Nannegari, VH, Haney, PM. GLUT1 colocalizes with the proteins of lactose synthetase and with endosomes in human mammary epithelial cells. Ped Res 2000; 47:296A (SPR poster presentation).
- 12. Riskin, A, Nannegari, VH, Haney, PM. An intact endosomal pathway is required for normal targeting of GFP-GLUT1 glucose transporter fusion proteins in living mouse mammary epithelial cells. Ped Res 2000; 47:296A (SPR poster symposium presentation).
- 13. Haney, PM. Intracellular targeting of GLUT1 in normal and neoplastic mammary epithelia. Proceedings of the Department of Defense Breast Cancer Research Program Meeting, 2000 (poster presentation).
- 14. An intact endosomal pathway is required for targeting of GFP-GLUT1 glucose transporter fusion proteins. Riskin A, Nannegari VH, Haney PM. Tenth Annual Meeting, International Society for Research in Human Milk and Lactation (poster presentation).
- 15. Prolactin-induced GLUT1 targeting to medial Golgi in human mammary epithelial cells. Riskin A, Nannegari VH, Haney PM. Tenth Annual Meeting, International Society for Research in Human Milk and Lactation (poster presentation).

Patents and licenses- none

Degrees- none

Development of cell lines, tissue or serum repositories- none

Informatics- none

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Lactose synthesis regulation: role of glucose transport, National Institutes of Health, FIRST Award, Principal Investigator, 9/20/98-9/19/03, \$70,000/yr., \$350,000, 1R29HD/DK34701.

Employment or research opportunities

Assistant Professor of Pediatrics, Baylor College of Medicine, 7/1/97-present

Conclusions

1. In normal CIT3 mammary epithelial cells, GLUT1 colocalizes with Golgi markers b-COP and a-mannosidase but not with the trans-Golgi marker Bodipy TR-ceramide.
2. GLUT1 targeting to Golgi is sensitive to Brefeldin A.
3. There are no higher molecular weight isoforms of GLUT1.
4. Glycosylation plays no role in GLUT1 targeting to Golgi.
5. There is no evidence that lactogenic hormones stimulate expression of a novel glucose transporter.
6. Forced weaning disrupts targeting of GLUT1 to Golgi even more rapidly than reported last year.
7. Golgi markers demonstrate heterogeneity in subcellular distribution in mammary gland.
8. Changes in Golgi markers with forced weaning suggest that changes in GLUT1 targeting during that time may reflect a dynamic reorganization process affecting all Golgi constituents.
9. Sec24 is differentially expressed in response to prolactin and hydrocortisone.
10. GLUT1-EBFP fusion protein offers the opportunity to study transporter targeting in living cells.
11. Golgi GLUT1 purified under non-denaturing conditions has an apparent molecular weight of 130 kD, suggesting that it may be associated with a protein of 70-90 kD.
12. MCF7 cancer cells exhibit very low rates of glucose transport.
13. MDA231 cells exhibit very high rates of glucose transport but do not appear to utilize GLUT1 for this purpose, suggesting expression of a novel transporter or "oncotransporter."
14. MDA231 cells sequester GLUT1 in an atypical-appearing intracellular compartment whether or not prolactin and hydrocortisone are present.
15. GLUT1 targeting in human mammary epithelial cells isolated from milk parallels findings in mouse cells.
16. Liposome-mediated transfection is highly efficient in mammary epithelial cells.
17. There is currently no evidence that a GLUT1-C-terminal binding protein regulates GLUT1 targeting in mammary gland.
18. GLUT1-EBFP fusion proteins are insufficiently sensitive to be useful.
19. GLUT1-DsRed1 fusion proteins are not useful due to artifactual targeting.
20. GLUT1-EGFP fusion proteins are valuable tools for understanding the dynamics of glucose transporter targeting in mammary epithelial cells.
21. Glucose transporter targeting is a dynamic process in both normal and neoplastic mammary epithelial cells.
22. An intact endosomal pathway is required for normal glucose transporter targeting.
23. Intracellular targeting of GLUT1 in neoplastic mammary epithelial cells is unrelated to intracellular targeting of GLUT1 to Golgi during lactation.
24. Mammary epithelial cells in culture do not express a variety of glucose transporters but do not express α -lactalbumin and do not synthesize lactose.
25. The expression of GLUT2 and GLUT3 is suppressed in neoplastic mammary epithelial cells.
26. Known glucose transporters do not account for glucose uptake in neoplastic

mammary epithelial cells.

So what? The results provide insight into the normal function of the mammary gland, the production of milk. Glucose transport into the Golgi may determine the rate of lactose synthesis and therefore milk production. Now that the mechanism of glucose transport into Golgi has been explained, it may be possible to develop ways to facilitate lactation and increase the prevalence and duration of breastfeeding. This will improve the health and neurodevelopment of the nation's children.

Through understanding the normal, we have the context for interpreting the abnormal. The results therefore have important implications for breast cancer. We identify targeting machinery unique to the neoplastic mammary epithelial cell that affects GLUT1, and may very well affect the targeting and activity of other cellular proteins in cancer cells. Furthermore, the results show that the known glucose transporters can not account for the glucose uptake of breast cancer cells, suggesting that a unique glucose transporter may be present, and may be an attractive therapeutic target.

Bibliography

See "Reportable Outcomes," pgs. 16-17.

Personnel Receiving Pay

Peter M. Haney, M.D., Ph.D., Principal Investigator
Theresa Joseph, Research Technician
Stella Tsang, Research Technician
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References

Bunn RC, Jensen MA, Reed BC, Protein interactions with the glucose transporter binding protein GLUT1CBP that provide a link between GLUT1 and the cytoskeleton. Mol Biol Cell 1999 Apr;10(4):819-32

CONTAINS UNPUBLISHED DATA

Appendices

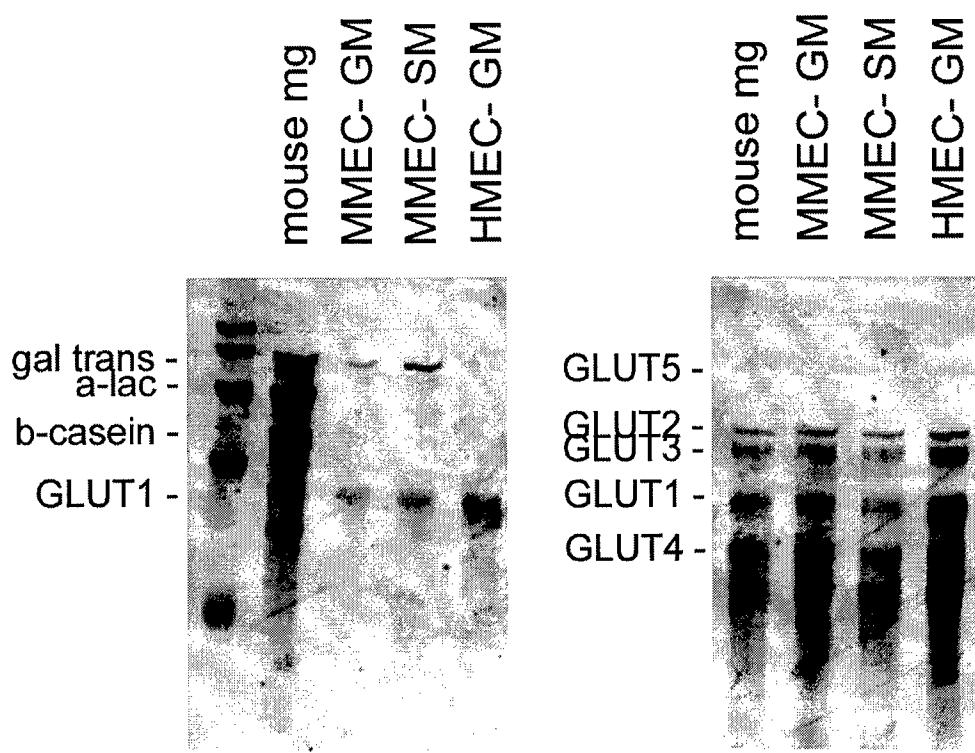


Figure 1. Ribonuclease protection assay. Left, mouse mammary gland, mouse mammary epithelial cells in growth medium and secretion medium, and human mammary epithelial cells in growth medium, were probed for mRNA for galactosyltransferase, α -lactalbumin, β -casein, and GLUT1. The results show that α -lactalbumin and β -casein are not expressed in culture, and that galactosyltransferase and GLUT1 are induced by prolactin and hydrocortisone. Right, mouse mammary gland, mouse mammary epithelial cells in growth medium and secretion medium, and human mammary epithelial cells in growth medium, were probed for mRNA for GLUT1-5. The results show that GLUT1 and GLUT4 dominate, smaller amounts of GLUT 3 and GLUT2 are seen, and no GLUT5 is detected.

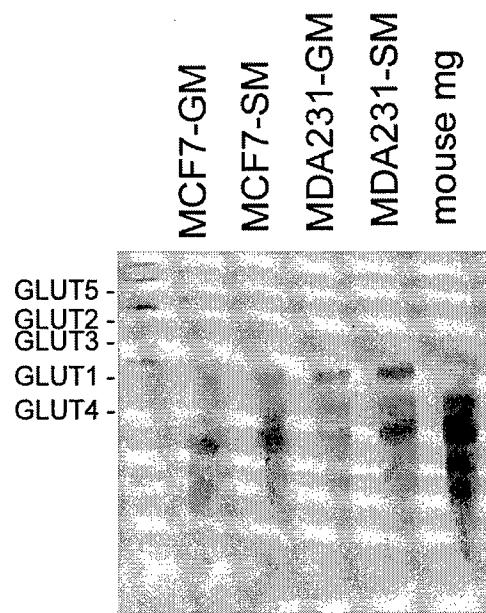


Figure 2. Ribonuclease protection assay. MCF7 and MDA231 human breast cancer cells in growth medium and secretion medium, and mouse mammary gland, were probed for mRNA for GLUT1-5. The results show that GLUT1 and GLUT4 dominate, and no GLUT2, GLUT3, or GLUT5 is detected.

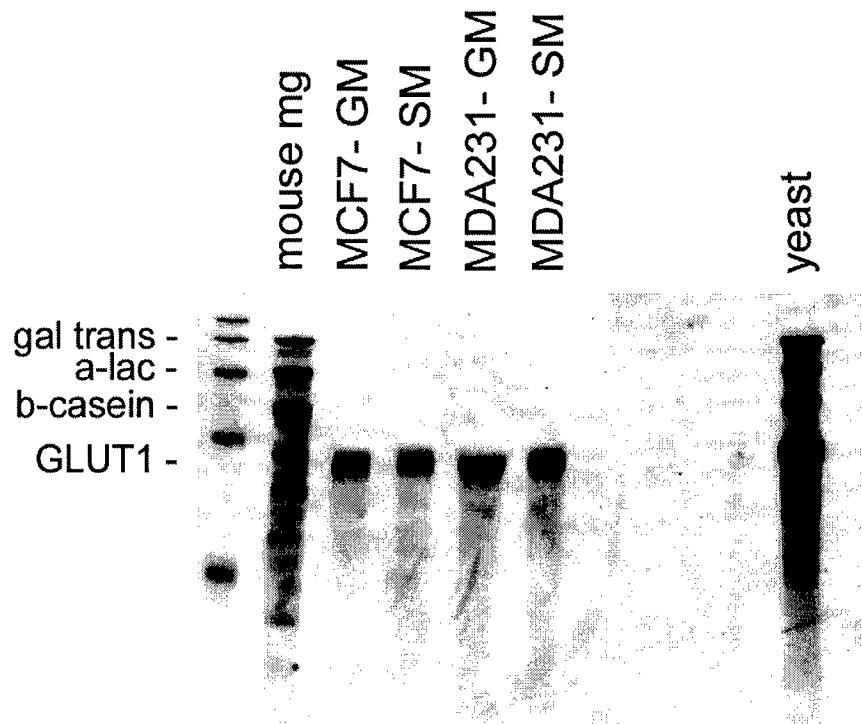


Figure 3. Ribonuclease protection assay. MCF7 and MDA231 human breast cancer cells in growth medium and secretion medium were probed for mRNA for galactosyltransferase, α -lactalbumin, β -casein, and GLUT1. Mouse mammary gland and yeast mRNA as a control are also shown. The results show that key proteins of lactation are not expressed in cancer cells, suggesting that the mechanism of sequestration of GLUT1 is independent from that operative during lactation.



Figure 4. Ribonuclease protection assay. CIT3 mouse mammary epithelial cells in growth medium and secretion medium, and with supranormal concentrations of prolactin (10/3), hydrocortisone (3/10), or both (10/10), were probed for mRNA for galactosyltransferase, α -lactalbumin, β -casein, and GLUT1. The results show no significant increase in GLUT1 or galactosyltransferase, and no expression whatsoever of α -lactalbumin and β -casein, despite increased hormone concentrations.

LOCALIZATION OF THE GLUT1 GLUCOSE TRANSPORTER TO
BREFELDIN A-SENSITIVE VESICLES OF DIFFERENTIATED CIT₃
MOUSE MAMMARY EPITHELIAL CELLS

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Abstract

Glucose is a precursor of lactose, the major carbohydrate and osmotic constituent of human milk, which is synthesized in the Golgi. The GLUT1 glucose transporter is the only glucose transporter isoform expressed in mammary gland. The hypothesis that lactogenic hormones induce GLUT1 and cause its localization to the Golgi of mammary epithelial cells was tested in CIT, mouse mammary epithelial cells. Treatment with prolactin and hydrocortisone caused a 15-fold induction of GLUT1 by Western blotting, but 2-deoxyglucose uptake decreased. Subcellular fractionation and density gradient centrifugation demonstrated enrichment of Golgi fractions with GLUT1. Lactogenic hormones enhanced GLUT1 glycosylation, but did not determine whether GLUT1 was targeted to plasma membrane or to Golgi. Confocal microscopy revealed that lactogenic hormones alter GLUT1 targeting from a plasma membrane pattern to a predominant perinuclear distribution with punctate scattering through the cytoplasm. GLUT1 is targeted to a compartment which is more sensitive to Brefeldin A than the compartments in which GM130 and β -COP reside. Targeting of GLUT1 to endosomes was specifically excluded. We conclude that prolactin and hydrocortisone induce GLUT1, enhance GLUT1 glycosylation, and cause glycosylation-independent targeting of GLUT1 to Brefeldin A-sensitive vesicles which may represent a subcompartment of cis-Golgi. These results demonstrate a hormonally regulated targeting mechanism for GLUT1 and are consistent with an important role for GLUT1 in the provision of substrate for lactose synthesis.

Introduction

Lactose is the major carbohydrate and the major osmotic constituent of human milk. Therefore, the amount of lactose synthesized determines the volume of milk produced by the lactating human mammary gland. The substrates for lactose synthesis are UDP-galactose and free glucose. Direct measurement of intracellular glucose concentration demonstrated that glucose transport into the mammary epithelial cell must be rate-limiting for lactose synthesis (Wilde and Kuhn, 1981). Lactose synthesis takes place within the Golgi (Keenan *et al.*, 1970) and is catalyzed by lactose synthetase, a complex of galactosyltransferase and the mammary gland specific protein α -lactalbumin. The mammary epithelial cell not only must transport glucose from the blood across the basal membrane into the cell, it must also deliver this glucose to the Golgi. The lactating mammary gland is unique in its requirement for free glucose within the Golgi. A Golgi specific glucose carrier protein accounting for Golgi glucose uptake during lactation was proposed 5 years before the cloning of GLUT1, the first member of the family of facilitated diffusion glucose transporter isoforms (White *et al.*, 1980).

The only isoform of the facilitated diffusion family of glucose transporters known to be expressed in mammary gland is GLUT1. In most cell types, GLUT1 is targeted to the plasma membrane. Neither GLUT1 nor any other glucose transporter isoform has been proven to target to the Golgi. In one study, subcellular fractionation and Western blotting of day-10 lactating rat mammary glands suggested that during lactation, GLUT1 may also be found in the Golgi (Madon *et al.*, 1990). However, in other studies (Camps *et al.*, 1994; Takata *et al.*, 1997) using microscopy, Golgi targeting of GLUT1 was not

observed in day 10-12 lactating rat mammary gland, although high and polarized expression of plasma membrane GLUT1 during lactation was seen. We have used epifluorescent and confocal microscopy and subcellular fractionation to demonstrate targeting of GLUT1 to Golgi of lactating mouse mammary gland. This is regulated by the developmental stage of the gland and the demand for milk, is most pronounced at the peak of lactation, and is reversibly diminished when pups are removed from the mother (Nemeth *et al.*, 2000).

In mammary epithelial cells, prolactin causes a shift in Golgi morphology from tubular to vesicular (Olivier-Bousquet, 1978). The purpose of our study was to test the hypothesis that prolactin and hydrocortisone cause the targeting of GLUT1 to Golgi in cultured mouse mammary epithelial cells. To provide multiple lines of evidence, methods included 2-deoxyglucose uptake assays, subcellular fractionation and density gradient centrifugation, and confocal immunofluorescent microscopy. The results indicate that in an established mammary epithelial cell line in culture, the GLUT1 glucose transporter is induced and targeted to Brefeldin A-sensitive vesicles distinct from Golgi stacks, as marked by EYFP-Golgi, GM130, and β -COP, under the influence of lactogenic hormones. These vesicles are neither endosomes nor lysosomes and may represent a Golgi-derived vesicle population important in lactose synthesis.

Materials and Methods

Antisera and reagents

A well characterized, highly specific rabbit polyclonal antiserum to human GLUT1, raised against synthetic peptide made up of the 16 C-terminal amino acids, was a kind gift of Dr. M. Mueckler (Washington University School of Medicine, St. Louis, MO). This antibody was affinity purified using the same synthetic peptide bound to thiopropyl sepharose (Parekh *et al.*, 1989) prior to use for immunoblotting or immunocytochemistry. Mouse monoclonal antibodies to rat β -COP (Sigma, St. Louis, MO) and rat GM130 (Transduction Laboratories, Lexington, KY) were used. Fluorescein-labeled goat anti-rabbit and Texas-red sheep anti-mouse antibodies were from ICN (Aurora, OH). Reagents were from Sigma unless otherwise specified.

Cell culture

CIT₃ cells are an established mouse mammary epithelial cell line (Toddywalla *et al.*, 1997), kindly supplied by Dr. M.C. Neville (University of Colorado School of Medicine, Denver, CO). Cells were maintained in growth medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM) with Ham's F12 (50:50), supplemented with 2% heat-inactivated fetal bovine serum (FBS), 10 μ g/ml insulin and 5 ng/ml epidermal growth factor (EGF). To differentiate the cells, growth medium lacking EGF but containing 3 μ g/ml prolactin and 3 μ g/ml hydrocortisone was used; this medium was designated "secretion medium". In certain experiments, cells were treated with 500 nM or 5 μ M Brefeldin A (Pauloin *et al.*, 1997) for 30 minutes at 37°C. Transferrin-Texas Red (Molecular Probes, Eugene, OR) was used at a concentration of 100 μ g/ml for 15 minutes at 37°C(Ghosh and Maxfield, 1995).

Transfection

EYFP-Golgi (Clontech, Palo Alto, CA) was expressed using cationic liposomes (LipoFectAmine™ Reagent, GibcoBRL, Rockville, MD). Transfection of 2 μ g DNA was performed on glass coverslips in 35 mm dishes containing 5×10^5 cells per plate, according to the manufacturer's instructions. After 48 hrs, cells were fixed and stained for GLUT1 using a Texas Red secondary antibody as described below.

2-Deoxyglucose uptake

[³H]2-Deoxyglucose uptake was measured as previously described (Tordjman *et al.*, 1989). Non-carrier-mediated uptake was measured in the presence of 20 μ M cytochalasin B and was subtracted from total 2-deoxyglucose uptake so that results are expressed as specific carrier-mediated uptake. Non-carrier-mediated uptake was less than 21% of total 2-deoxyglucose uptake under all circumstances. Statistical significance of the difference between the groups was calculated using Student's t-test for independent samples.

Subcellular fractionation and density gradient centrifugation

CIT₃ cells were rinsed twice with ice-cold PBS and once with ice-cold homogenization buffer. Cells were harvested using a rubber policeman. The pellet was resuspended in a small volume of homogenization buffer and homogenized with five strokes in a tight-fitting Dounce homogenizer. After one centrifugation at 3,000 g for 10 minutes at 4°C, the supernatant was centrifuged at 17,000 g for 10 minutes at 4°C. This supernatant was centrifuged at 100,000 g for 30 minutes at 4°C. The 17,000 g pellet was resuspended and subjected to density gradient centrifugation in a self-generating iodixanol density gradient (10%-37%) at 180,000 g for 3 hours at 4°C. Twenty fractions

were collected from the top (Labconco Auto-densi Flow, Kansas City, MO) and analyzed. Alkaline phosphatase (Langridge-Smith *et al.*, 1998) and galactosyl transferase (Graham, 1993) were assayed as described.

Western blotting

Samples were prepared and subjected to standard SDS-PAGE on 10% gels as previously described (Haney *et al.*, 1991). Proteins were immobilized on nitrocellulose by wet transfer. The peptide affinity purified GLUT1 antibody described above (1 μ g/ml in 5% nonfat dry milk in PBS) was used as primary antibody. Secondary antibody was HRP-linked donkey anti-rabbit antibody (Amersham, Piscataway, NJ), and signal was detected using ECL-PlusTM (Amersham). Quantitative differences in signal strength were measured using a STORM analyzer (Molecular Dynamics, Sunnyvale, CA).

Immunocytochemistry

Cells were grown on glass coverslips, washed with PBS, and fixed in 4% paraformaldehyde in serum-free medium for 20 minutes at room temperature. Cells were then treated with 100 mM glycine in PBS for 10 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes. Cells were blocked with 2% horse serum in PBS for 15 minutes. Treatment with primary antibody in 0.1% horse serum in PBS was overnight at 4°C. GLUT1 antibody was at 6 μ g/ml, GM130 antibody was at 5 μ g/ml, and β -COP antibody was at 1:80. After three 10-minute washes with PBS, secondary antibody, diluted 1:100 in 0.1% horse serum, was added for 30 minutes at room temperature. FITC-conjugated goat anti-rabbit antibody and Texas Red conjugated rabbit anti-mouse antibody were used. In experiments with cells transfected with EYFP-Golgi, Texas Red

conjugated goat anti-rabbit antibody was used. Coverslips were then washed three times with PBS and mounted in ProLong anti-fade medium (Molecular Probes, Eugene, OR) on glass slides for examination using a Molecular Dynamics MultiprobeTM 2010 inverted confocal laser scanning microscope. The images are shown as acquired; no enhancement or intensification was performed.

Results

Lactogenic hormones cause intracellular targeting of GLUT1

CIT₃ cells are an established mouse mammary epithelial cell line selected from Comma-1-D cells for their ability to express β -casein, form tight junctions, and exhibit polarized transport in response to treatment with prolactin and hydrocortisone (Toddywalla *et al.*, 1997). The total cellular content of GLUT1 in CIT₃ cells rose 15-fold in response to prolactin and hydrocortisone (Fig. 1A). Paradoxically, the 2-deoxyglucose uptake of these cells fell 70% in response to these hormones (Fig. 1B). In other words, cells maintained in secretion medium exhibited only a small fraction of the plasma membrane glucose transport activity predicted from their GLUT1 content. This suggested a major effect of lactogenic hormones either on the specific activity of the transporter or on the amount of transporter in the plasma membrane.

To distinguish between these possibilities, subcellular fractionation was carried out. Pellets from the 17,000 g and 100,000 g centrifugations were enriched in Golgi and plasma membrane, respectively, as indicated by assays of galactosyltransferase and alkaline phosphatase. Each marker was enriched 2.3-fold (for galactosyl transferase in

17,000 g pellet vs. homogenate, and for alkaline phosphatase in the 100,000 g pellet vs. homogenate) in the corresponding fraction. Compared to the enrichment observed for these markers, the enrichment of GLUT1 was much greater. GLUT1 was enriched 19.0-fold in the 100,000 g pellet, compared to homogenate, from cells maintained in growth medium, and was enriched 11.0-fold in the 17,000 g pellet, compared to homogenate, from cells grown in secretion medium (Fig. 2). Cells grown in growth medium showed predominance of GLUT1 in the plasma membrane-enriched fraction, while secretion medium caused predominance of GLUT1 targeting in the Golgi-enriched fraction (Fig. 2). The relative molecular mass of GLUT1 in cells grown in secretion medium was slightly higher than in growth medium, 53 kDa compared to 50 kDa, suggesting the possibility of a second glucose transporter closely related to GLUT1. Therefore, homogenate, 17,000 g pellets, and 100,000 g pellets from cells maintained in both growth medium and secretion medium were subjected to enzymatic deglycosylation. In all cases, the deglycosylated protein had an identical relative molecular mass of 37 kDa, consistent with GLUT1 (Fig. 2).

To confirm that the GLUT1 contained in the 17,000 g pellet was targeted to Golgi, iodixanol density gradient centrifugation was carried out. GLUT1 was concentrated in the low-density fractions (1.05-1.08 g/cm³), consistent with Golgi (Fig. 3). The difference in the absolute amount of GLUT1 targeting to Golgi between cells grown in growth medium and in secretion medium was consistent with that seen in Fig. 2.

The results exclude lysosomes, which are found at a density of 1.10-1.15 g/cm³ in low density medium (Spector *et al.*, 1997), as the intracellular compartment to which GLUT1 is targeted.

Confocal immunofluorescent microscopy of cells maintained in growth medium demonstrated a plasma membrane pattern for GLUT1 (Fig. 4A). In contrast, consistent with the results of subcellular fractionation and density gradient centrifugation, cells maintained in secretion medium exhibited much less plasma membrane signal. Rather, these cells showed a significant intracellular signal for GLUT1, distributed in a perinuclear pattern and in an intense punctate pattern throughout the cytoplasm (Fig. 4D). The trans-Golgi marker BODIPY-TR ceramide (Pagano *et al.*, 1991) demonstrated the expected punctate staining both in cells grown in growth medium (Fig. 4B) and in secretion medium (Fig. 4E). There was no colocalization of GLUT1 and the trans-Golgi marker in either growth medium (Fig. 4C) or secretion medium (Fig. 4F).

Brefeldin A was used to further dissect Golgi targeting. Cells maintained in growth medium and subjected to 500 nM Brefeldin A showed distribution of the trans-Golgi marker BODIPY-TR ceramide throughout the cytoplasm (Fig. 5B). The plasma membrane targeting of GLUT1 was not affected (Fig. 5A), and there was no specific colocalization of GLUT1 and BODIPY-TR ceramide (Fig. 5C). BODIPY-TR ceramide was also distributed throughout the cytoplasm in cells maintained in secretion medium and exposed to Brefeldin A (Fig. 5E), and, again, there was no specific colocalization of GLUT1 and BODIPY-TR ceramide (Fig. 5F). However, GLUT1 targeting to the perinuclear area was much less prominent; rather, the GLUT1 signal was diffusely distributed in a punctate pattern.

GM130 is a Golgi matrix protein associated with cis-Golgi membranes (Nakamura *et al.*, 1995), and demonstrated a classical Golgi appearance in cells maintained in secretion medium (Fig. 6B). Its targeting demonstrated a dose-dependent effect of Brefeldin A. At a concentration of 500 nM, the classical Golgi appearance remained, although a small amount of signal became visible throughout the cell (Fig 6E). In contrast, at 5 μ M Brefeldin A, GM130 was distributed throughout the cell, with minimal perinuclear prominence (Fig. 6H). The dispersion of GLUT1 was similar at 500 nM (Fig 6D) and 5 μ M (Fig 6G) Brefeldin A. Apparent colocalization of GLUT1 and GM130 was observed only at 5 μ M Brefeldin A.

EYFP-Golgi contains an 85 amino acid segment from β -galactosyltransferase which confers classical medial-Golgi targeting (Fig. 7B). Its targeting was more sensitive to Brefeldin A than was the targeting of GM130, as the classical Golgi appearance transformed to a reticular structure (Fig 7E) at a Brefeldin A concentration of 500 nM. A very small degree of apparent colocalization of EYFP-Golgi and GLUT1 was observed in secretion medium (Fig 7C), although a proximity effect as opposed to genuine colocalization can not be excluded. In contrast, in the presence of Brefeldin A there was no colocalization of EYFP-Golgi with GLUT1.

The predominantly perinuclear distribution of β -COP was not significantly affected by Brefeldin A (Figs 8B,E). GLUT1 was again found in a perinuclear distribution and in a punctate pattern throughout the cytoplasm (Figs 8A,D), with a shift from the perinuclear area under the influence of Brefeldin A. There was no colocalization

of GLUT1 and β -COP in secretion medium. However, Brefeldin A enhanced colocalization of GLUT1 with β -COP (Fig. 8F).

This indicates that lactogenic hormones present in secretion medium cause substantial GLUT1 targeting to Brefeldin A-sensitive vesicles distinct from those subcompartments marked by BODIPY-TR ceramide, GM130, EYFP-Golgi, and β -COP. Brefeldin A appeared to cause mixing of the GLUT1 subcompartment with the GM130 and β -COP subcompartments, but not the subcompartments marked by BODIPY-TR ceramide and EYFP-Golgi.

To exclude the possibility that GLUT1 was targeted to endosomes, cells were briefly labeled with Transferrin-Texas Red (Ghosh and Maxfield, 1995). Endosomal staining was completely distinct from GLUT1 (Fig. 9A-C). Brefeldin A did not cause colocalization of GLUT1 and endosomes (Fig. 9D-F). The results exclude endosomes as a potential localization of GLUT1.

Since GLUT1 demonstrated a plasma membrane targeting pattern in cells maintained in growth medium, there was no colocalization of GLUT1 with these markers of intracellular compartments (data not shown).

Discussion

GLUT1 is the only member of the facilitated diffusion glucose transporter family known to be expressed in mammary gland. However, in the many cell types in which it is expressed, GLUT1 is a plasma membrane protein, and is responsible for the basal uptake of glucose into most cells. None of the isoforms of the glucose transporter family

is considered to be a Golgi resident, although a six-amino acid portion of GLUT4 does confer targeting to the trans-Golgi network and insulin-sensitive translocation to the plasma membrane in fat and muscle cells (Haney *et al.*, 1995). Furthermore, lactose synthesis within the Golgi is a unique function of differentiated mammary epithelial cells; no other cell type requires free glucose within the Golgi.

Several investigators have studied GLUT1 expression in the lactating mammary gland, with conflicting results described above. We have demonstrated that GLUT1 is targeted to Golgi, as defined by a low-density intracellular compartment substantially colocalizing with β -COP, in lactating mouse mammary gland (Nemeth *et al.*, 2000). The purpose of the experiments reported here was to test, in mammary epithelial cells in culture, whether the subcellular trafficking of GLUT1 is hormonally regulated in a manner consistent with an important role for GLUT1 in the provision of substrate for lactose synthesis.

Prolactin and hydrocortisone trigger differentiation of mammary epithelial cells. Total cellular GLUT1 levels increased 15-fold in response to this treatment. Importantly, consistent results from two independent methods, subcellular fractionation followed by density gradient centrifugation, and confocal immunofluorescent microscopy, indicate that prolactin and hydrocortisone cause GLUT1 to be targeted not to the plasma membrane, as it is in most cells, but to a Brefeldin A-sensitive vesicular compartment. The subcellular fractionation and density gradient characterization include expected degrees of enrichment in appropriate marker enzymes and demonstrate that the GLUT1-enriched fractions exhibit a very low density (1.05-1.08 g/cm³) fraction of the light

mitochondrial (17,000 g) pellet, consistent with Golgi localization. Since lysosomes are found at a density of 1.10-1.15 g/cm³ in low density medium (Spector *et al.*, 1997), they are excluded as the intracellular compartment to which GLUT1 is targeted.

We have not yet examined whether the turnover of GLUT1 is altered under these conditions, but adequate time to achieve a new steady state was insured by allowing four days of exposure to prolactin and hydrocortisone before assessing changes in steady-state distributions. This eliminates the possibility that altered turnover alone is responsible for changes in GLUT1 targeting.

Brefeldin A causes the rapid and reversible retrograde movement of Golgi resident proteins back to the endoplasmic reticulum (Klausner *et al.*, 1992). In most cells, trans-Golgi network proteins respond to Brefeldin A by fusing with early endosomes; the hybrid organelles are stable and tubulovesicular in contrast to the Golgi, which disappears (Lippincott-Schwartz *et al.*, 1991). However, polarized epithelial cells respond differently to Brefeldin A. In MDCK cells, BFA promoted extensive tubulation of the trans-Golgi network while the medial Golgi marker alpha-mannosidase II was not affected (Wagner *et al.*, 1994). As was recently demonstrated in mammary epithelial cells from lactating rabbit, Brefeldin A (50 nM) caused dissociation of trans-Golgi but not medial Golgi marker enzymes (Pauloin *et al.*, 1997). In our study of cells in culture, similar dose-dependent effects of Brefeldin A were seen, but at somewhat higher concentrations than observed by Pauloin *et al.* in rabbit mammary gland. We exposed cells to Brefeldin A for 30 minutes, as originally reported (Pagano *et al.*, 1991). Pauloin *et al.* concluded that rabbit mammary epithelial cells equilibrated after 30 minutes of Brefeldin A exposure (Pauloin *et al.*, 1997).

Thus, under the influence of prolactin and hydrocortisone, GLUT1 is sequestered within the cell, accounting for the paradoxical decrease in plasma membrane glucose transport activity despite increased GLUT1 levels caused by these hormones. This leads to the conclusion that GLUT1 is diverted from normal sorting pathways to a Brefeldin A-sensitive compartment. This compartment represents neither endosomes nor lysosomes. In AtT20 cells, Brefeldin A at 1 μ g/ml converted Golgi stacks to clusters of tubules and vesicles but had no detectable effect on early endosomes, while concentrations as high as 20 μ g/ml Brefeldin A had no effect on late endosomes/prelysosomes (Tooze and Hollinshead, 1992). In CIT3 cells, GLUT1 consistently demonstrated a loss in perinuclear compartments at only 500 nM Brefeldin A. While this GLUT1-containing compartment may represent Golgi-derived vesicles, it lacks the Golgi markers GM130, EYFP-Golgi, and β -COP. The discrepancy between these results and our results in the intact gland may be due to limitations of the cells in culture, which may not express all machinery required for authentic targeting of GLUT1. Further work is required to determine whether other factors, such as additional hormones or extracellular matrix, might result in colocalization of GLUT1 with GM130, EYFP-Golgi, and β -COP in these cells.

The results suggest a tissue- and developmental stage-specific Golgi targeting mechanism for GLUT1. The structural determinants of targeting of resident proteins to Golgi are controversial. For certain glycosyltransferases, the transmembrane spanning domain or specific amino acid motifs within it have seemed important (Nilsson and Warren, 1994). However, examination of a large number of cloned glycosyltransferases

does not reveal a common retention signal (Keenan, 1998). Alternative hypotheses include the formation of non-mobile protein oligomers and the influence of the high cholesterol content of Golgi membranes on mobility of resident Golgi enzymes (Munro, 1998). Although glycosylation serves to direct protein targeting to apical membrane of polarized epithelial cells under certain circumstances (Gut *et al.*, 1998), it has not been linked with targeting of proteins to Golgi. In fact, a mutant form of GLUT1 lacking the N-glycosylation site shows intracellular targeting (Asano *et al.*, 1993). Although we observed enhanced glycosylation of GLUT1 in response to prolactin and hydrocortisone, deglycosylation experiments reported here found no evidence that differential glycosylation is responsible for Golgi targeting. In the context of our primitive general understanding of the determinants of protein targeting to Golgi, it is not surprising that the molecular basis of the hormonally regulated Golgi targeting of GLUT1 is unclear. Hormonal regulation of the process of Golgi targeting of GLUT1 suggests a flexibility of the Golgi targeting machinery that has not previously been appreciated. The results suggest a tissue-specific GLUT1 sorter is active during lactation. Further study may reveal mechanisms regarding GLUT1 targeting that are relevant to other Golgi proteins as well.

Deglycosylation experiments (Fig. 2) showed that the differences in relative molecular mass of GLUT1 between cells maintained in growth medium and in secretion medium need not be attributed to expression of a distinct glucose transporter isoform containing the GLUT1 antigenic determinant. However, the results do not exclude the previously suggested (Madon *et al.*, 1990) possibility that a novel transporter, yet to be identified, resides in the Golgi.

The data were developed in an established mammary epithelial cell line grown on tissue culture plastic, in the absence of reconstituted basement membrane. The subcellular fractionation and iodixanol density gradient centrifugation procedures resulted in enriched, not purified, fractions, which nevertheless demonstrated altered GLUT1 targeting, confirmed by confocal immunofluorescent microscopy. Taken together, the data demonstrate specific changes in glucose transporter targeting, and support the conclusion that important cellular mechanisms must underlie them, in this simplest possible, but artificial, system. Studies in more physiologically relevant model systems are under way. Future work will focus on understanding the mechanism and regulation of GLUT1 targeting to Golgi during lactation.

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References

ASANO T, TAKATA K, KATAGIRI H, ISHIHARA H, INUKAI K, ANAI M, HIRANO H, YAZAKI Y, OKA Y, 1993. The role of N-glycosylation in the targeting and stability of GLUT1 glucose transporter. *FEBS Lett* **324**:258-261.

CAMPS M, VILARO S, TESTAR X, PALACIN M, ZORZANO A, 1994. High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. *Endocrinology* **134**:924-934.

GHOSH RN, MAXFIELD FR, 1995. Evidence for nonvectorial, retrograde transferrin trafficking in the early endosomes of HEp2 cells. *J Cell Biol* **128**:549-561.

GRAHAM JM, 1993. The identification of subcellular fractions from mammalian cells. In: Graham JM, Higgins JA, eds. *Biomembrane protocols: I. Isolation and analysis*. Totowa, NJ: Humana Press, 1-18.

GUT A, KAPPELER F, HYKA N, BALDA MS, HAURI HP, MATTER K, 1998. Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins. *EMBO J* **17**:1919-1929.

HANEY PM, LEVY MA, STRUBE MS, MUECKLER M, 1995. Insulin-sensitive targeting of the GLUT4 glucose transporter in L6 myoblasts is conferred by its COOH-terminal cytoplasmic tail. *J Cell Biol* **129**:641-658.

HANEY PM, SLOT JW, PIPER RC, JAMES DE, MUECKLER M, 1991. Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J Cell Biol* **114**:689-699.

KEENAN TW, 1998. Biochemistry of the Golgi apparatus. *Histochem Cell Biol* **109**:505-516.

KEENAN TW, MORRE DJ, CHEETHAM RD, 1970. Lactose synthesis by a Golgi apparatus fraction from rat mammary gland. *Nature* **228**:1105-1106.

KLAUSNER RD, DONALDSON JG, LIPPINCOTT-SCHWARTZ J, 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J Cell Biol* **116**:1071-1080.

LANGRIDGE-SMITH JE, FIELD M, DUBINSKY WP, 1998. Isolation of transporting plasma membrane vesicles from bovine tracheal epithelium. *Biochim. Biophys. Acta* **731**:318-328.

LIPPINCOTT-SCHWARTZ J, YUAN L, TIPPER C, AMHERDT M, ORCI L, KLAUSNER RD, 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell* **67**:601-616.

MADON RJ, MARTIN S, DAVIES A, FAWCETT HA, FLINT DJ, BALDWIN SA, 1990. Identification and characterization of glucose transport proteins in plasma

membrane- and Golgi vesicle-enriched fractions prepared from lactating rat mammary gland. *Biochem. J.* **272**:99-105.

MUNRO S, 1998. Localization of proteins to the Golgi apparatus. *Trends Cell Biol* **8**:11-15.

NAKAMURA N, RABOUILLE C, WATSON R, NILSSON T, HUI N, SLUSAREWICZ P, KREIS TE, WARREN G, 1995. Characterization of a cis-Golgi matrix protein, GM130. *J Cell Biol* **131**:1715-1726.

NEMETH BA, TSANG SWY, GESKE RS, HANEY PM, 2000. Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland. *Pediatr Res* **47**:644-650.

OLLIVIER-BOUSQUET M, 1978. Early effects of prolactin on lactating rabbit mammary gland. Ultrastructural changes and stimulation of casein secretion. *Cell Tissue Res* **187**:25-43.

PAGANO RE, MARTIN OC, KANG HC, HAUGLAND RP, 1991. A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J Cell Biol* **113**:1267-1279.

PAREKH BS, SCHWIMMBECK PW, BUCHMEIER MJ, 1989. High efficiency immunoaffinity purification of anti-peptide antibodies on thiopropyl sepharose immunoadsorbants. *Pept Res* **2**:249-252.

PAULOIN A, DELPAL S, CHANAT E, LAVIALLE F, AUBOURG A, OLLIVIER-BOUSQUET M, 1997. Brefeldin A differently affects basal and prolactin-stimulated milk protein secretion in lactating rabbit mammary epithelial cells. *Eur J Cell Biol* **72**:324-336.

SPECTOR DL, GOLDMAN RD, LEINWAND LA, 1997. *Cells: a laboratory manual.*, p. 34.5, CSHL Press, Plainview, New York.

TAKATA K, FUJIKURA K, SUZUKI M, SUZUKI T, HIRANO H, 1997. GLUT1 glucose transporter in the lactating mammary gland in the rat. *Acta Histochem. Cytochem.* **30**:623-628.

TODDYWALLA VS, KARI FW, NEVILLE MC, 1997. Active transport of nitrofurantoin across a mouse mammary epithelial monolayer. *J. Pharmacol. Exp. Ther.* **280**:669-676.

TOOZE J, HOLLINSHEAD M, 1992. In AtT20 and HeLa cells brefeldin A induces the fusion of tubular endosomes and changes their distribution and some of their endocytic properties. *J Cell Biol* **118**:813-830.

TORDJMAN KM, LEINGANG KA, JAMES DE, MUECKLER MM, 1989. Differential regulation of two distinct glucose transporter species expressed in 3T3-L1 adipocytes: effect of chronic insulin and tolbutamide treatment. *Proc Natl Acad Sci U S A* **86**:7761-7765.

WAGNER M, RAJASEKARAN AK, HANZEL DK, MAYOR S, RODRIGUEZ-BOULAN E, 1994. Brefeldin A causes structural and functional alterations of the trans-Golgi network of MDCK cells. *J Cell Sci* **107** (Pt 4):933-943.

WHITE MD, KUHN NJ, WARD S, 1980. Permeability of lactating-rat mammary gland Golgi membranes to monosaccharides. *Biochem. J.* **190**:621-624.

WILDE CJ, KUHN NJ, 1981. Lactose synthesis and the utilisation of glucose by rat mammary acini. *Int. J. Biochem.* **13**:311-316.

Figure legends

Figure 1. Total cellular GLUT1 content and 2-deoxyglucose uptake of CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Secretion medium, which contains prolactin and hydrocortisone, caused a 15-fold increase in total cellular GLUT1 (A); this was associated with a paradoxical decrease in 2-deoxyglucose uptake (B). Samples from cells grown in growth medium (60 µg protein, lane 1) and secretion medium (20 µg protein, lane 2) were analyzed by SDS-PAGE and Western blotting. 2-Deoxyglucose uptake reflects glucose transport across the plasma membrane and was measured as described in Materials and Methods. Results are means +/- S.E.M. for six determinations. Student's t-test for independent samples resulted in a p value of 0.003 for the difference between groups in 2-deoxyglucose uptake.

Figure 2. Subcellular distribution and PNGase F digestion of GLUT1 in CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Subcellular fractions were prepared and 10 µg aliquots were analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. In growth medium, GLUT1 was most highly enriched in the 100,000 g pellet (lane 5), but in secretion medium GLUT1 was most highly enriched in the 17,000 g pellet (lane 9). The increase in signal in the homogenate between growth medium (lane 1) and secretion medium (lane 7) is consistent with the result in Figure 1A. The identical relative molecular mass of GLUT1 from all PNGase F treated samples, 37 kDa, indicates that the slightly higher apparent M_r of GLUT1 in

secretion medium, 53 kDa, compared to growth medium, 50 kDa, is due to differential glycosylation. Note also that targeting to subcellular fractions is not a function of the degree of glycosylation. Growth medium, lanes 1-6; secretion medium, lanes 7-12.

Figure 3. Iodixanol density gradient centrifugation of the 17,000 g pellet from CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Signal was found in only the least dense fractions, indicating that, under both conditions, the GLUT1 content of the 17,000 g pellet reflected its presence in the Golgi. Samples of 10 µg protein from iodixanol density gradient fractions of cells grown in growth medium (upper panel) and secretion medium (lower panel) were analyzed by SDS-PAGE and Western blotting as described in Materials and Methods.

Figure 4. Confocal images of GLUT1 (A,D) and the trans-Golgi marker BODIPY-TR ceramide (B,E) in CIT₃ cells maintained for 4 days in growth medium (A-C) and in secretion medium (D-F). Cells were fixed and stained, and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels; GLUT1 is shown in green, BODIPY-TR ceramide is shown in red, and areas of coincident staining appear yellow. Bar, 2 µm. Little overlap is observed. GLUT1 displayed a predominant plasma membrane pattern in growth medium, but in secretion medium, GLUT1 was distributed in a perinuclear pattern and in a punctate pattern throughout the cytoplasm.

Figure 5. Confocal images of GLUT1 (A, D) and BODIPY-TR ceramide (B, E) in CIT₃ cells maintained for 4 days in growth medium (A-C) and secretion medium (D-F) in the presence of Brefeldin A. Cells were treated for 30 minutes with 500 nM Brefeldin A. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; BODIPY-TR ceramide is shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. Brefeldin A completely disrupted BODIPY-TR ceramide targeting, but did not cause colocalization of GLUT1 and BODIPY-TR ceramide.

Figure 6. Confocal images of GLUT1 (A, D, G) and GM130 (B, E, H) in CIT₃ cells maintained for 4 days in secretion medium in the absence and presence of Brefeldin A. Cells were treated for 30 minutes with 500 nM Brefeldin A (D, E, F) or 5 μ M Brefeldin A (G, H, I). Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F, I) are superimpositions of the left and middle panels. GLUT1 is shown in green; GM130 is shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. Brefeldin A at 500 nM maximally disrupted GLUT1 targeting, but had only a subtle effect on GM130. Brefeldin A at 500 nM virtually completely disrupted GM130 targeting, and some apparent colocalization was seen.

Figure 7. Confocal images of GLUT1 (A, D) and EYFP-Golgi, a fusion of yellow fluorescent protein and the targeting domain of galactosyltransferase (B, E) in CIT₃ cells maintained for 4 days in secretion medium in the absence and presence of Brefeldin A. Cells were treated for 30 minutes with 500 nM Brefeldin A. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in red; EYFP-Golgi is shown in green, and areas of coincident staining appear yellow. Bar, 2 μ m. A small degree of possible colocalization of GLUT1 and EYFP-Golgi is seen in secretion medium (C), but not in the presence of 500 nM Brefeldin A (F).

Figure 8. Confocal images of GLUT1 (A, D) and β -COP (B, E) in CIT₃ cells maintained for 4 days in secretion medium in the absence and presence of Brefeldin A. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; β -COP is shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. Cells (D-F) were treated for 30 minutes with 500 nM Brefeldin A. Although there was no colocalization of GLUT1 and β -COP in secretion medium (C), treatment with Brefeldin A did result in apparent colocalization (F).

Figure 9. Confocal images of GLUT1 (A, D) and transferrin-Texas Red (B, E) in CIT₃ cells maintained for 4 days in secretion medium in the absence and presence of Brefeldin A. Cells were treated for 30 minutes with 500 nM Brefeldin A. Cells were fixed and

stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; transferrin-Texas Red is shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. No colocalization of GLUT1 and transferrin-Texas Red is seen, indicating that GLUT1 is not targeted to endosomes.

Figure 1

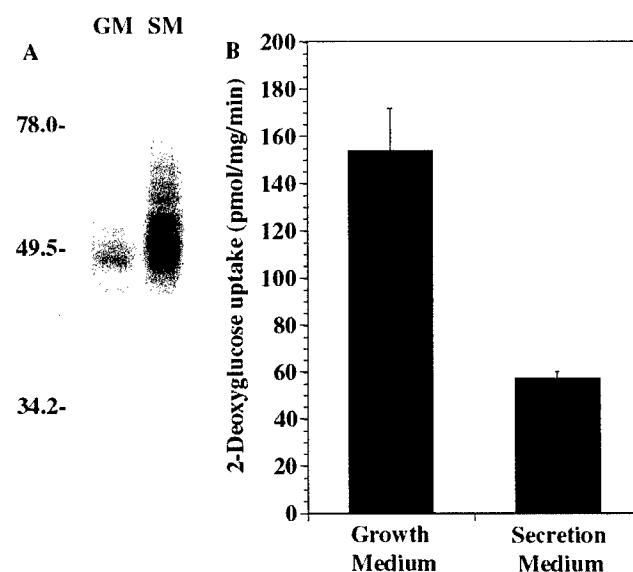


Figure 2

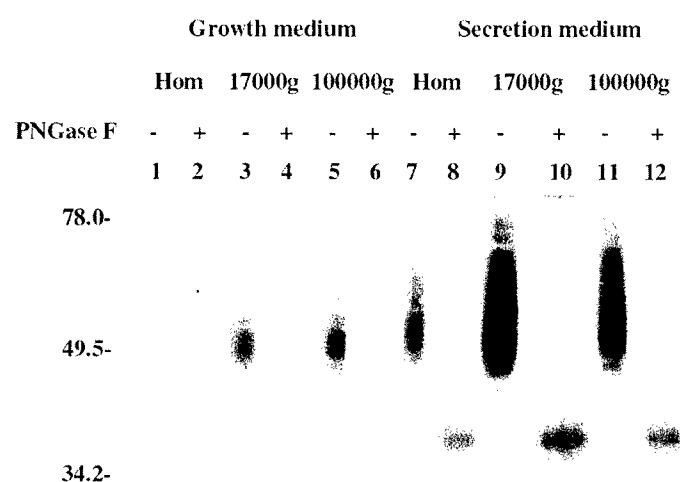


Figure 3

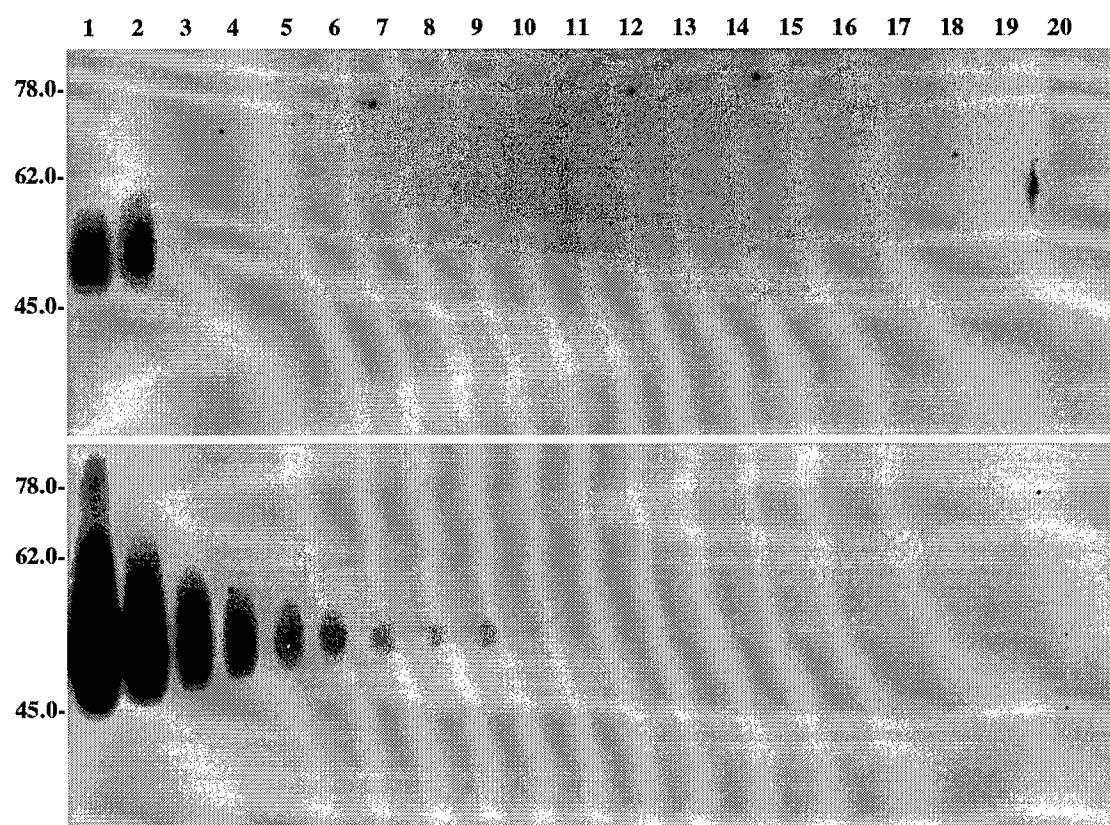


Figure 4

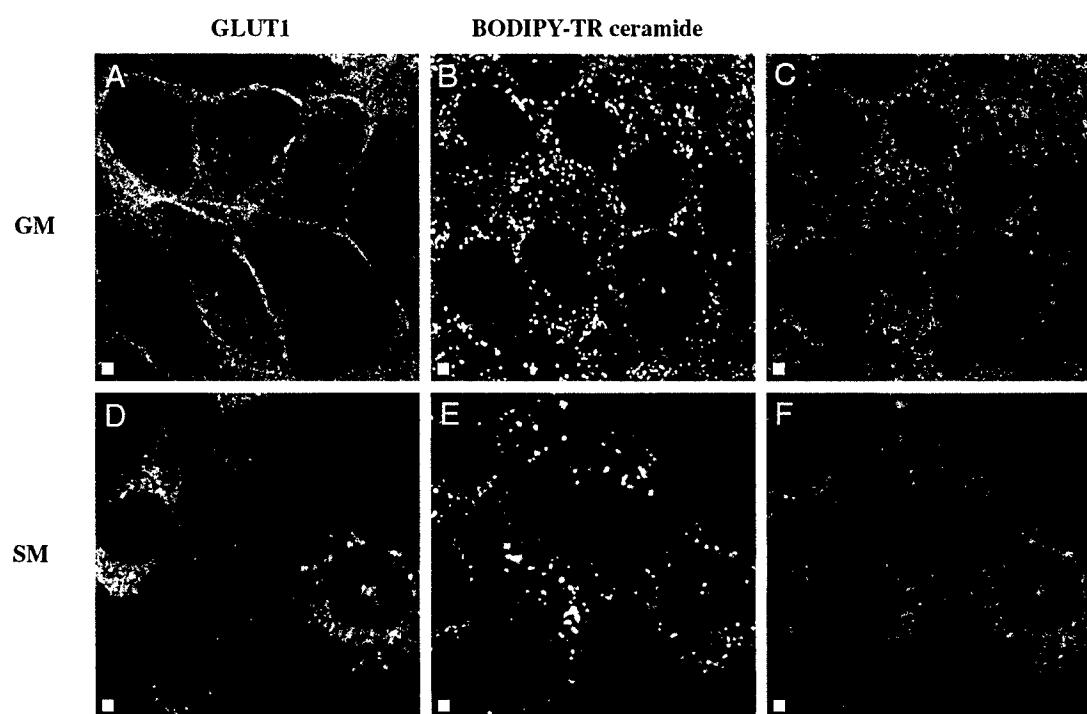


Figure 5

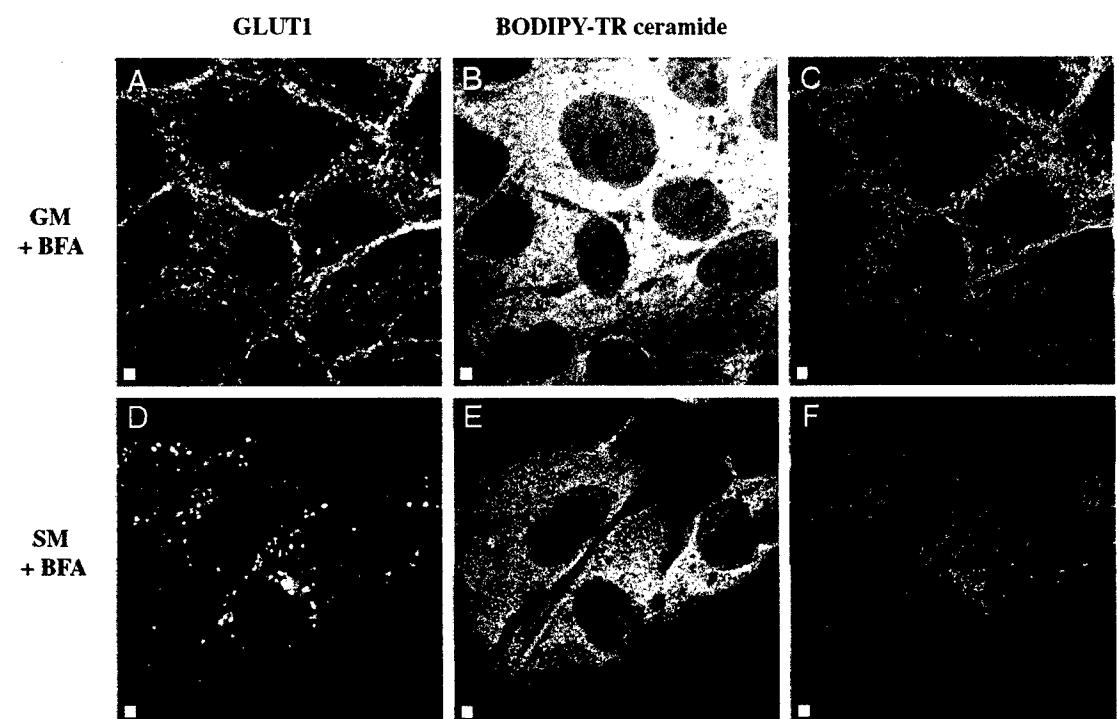


Figure 6

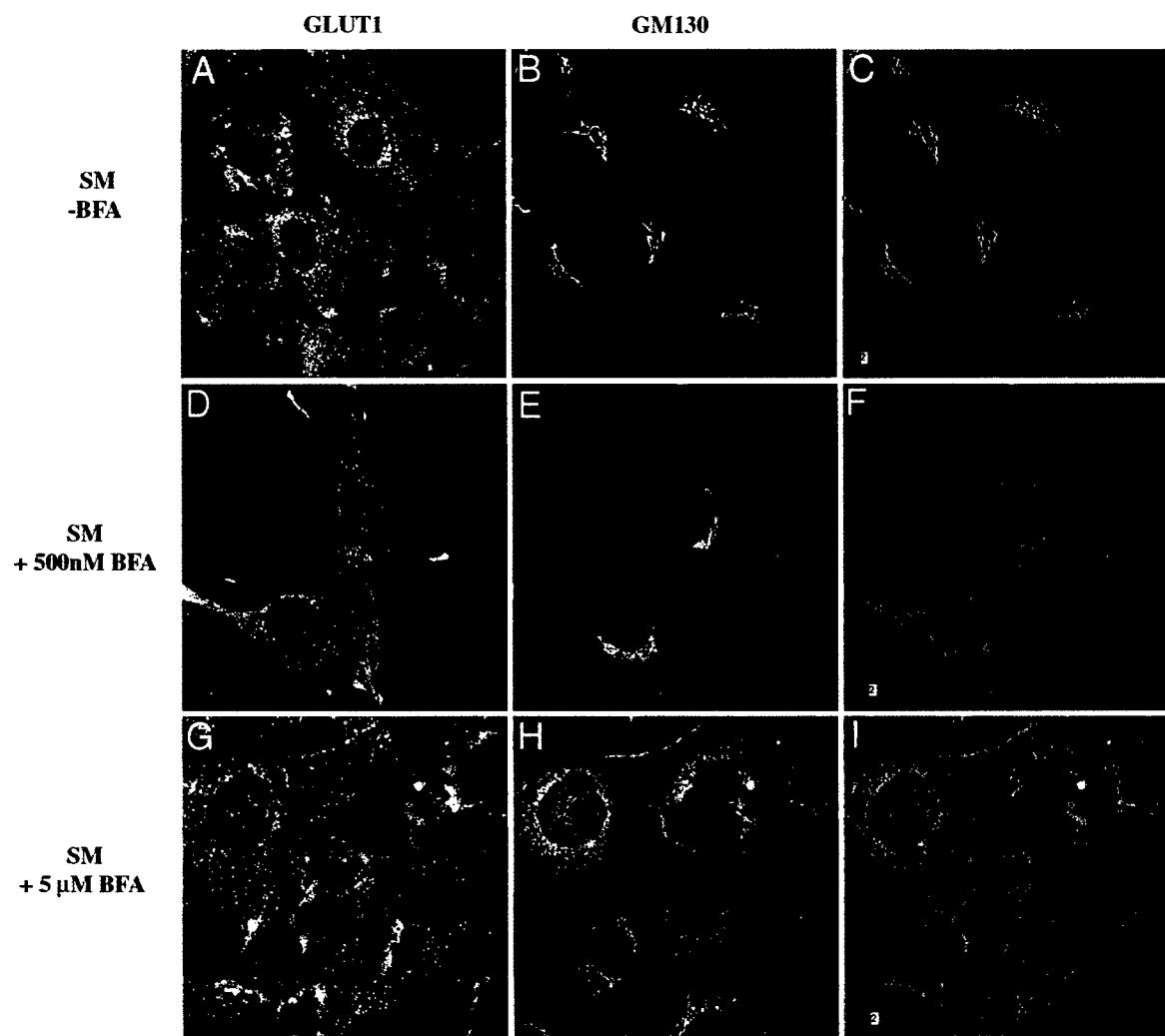


FIGURE 7

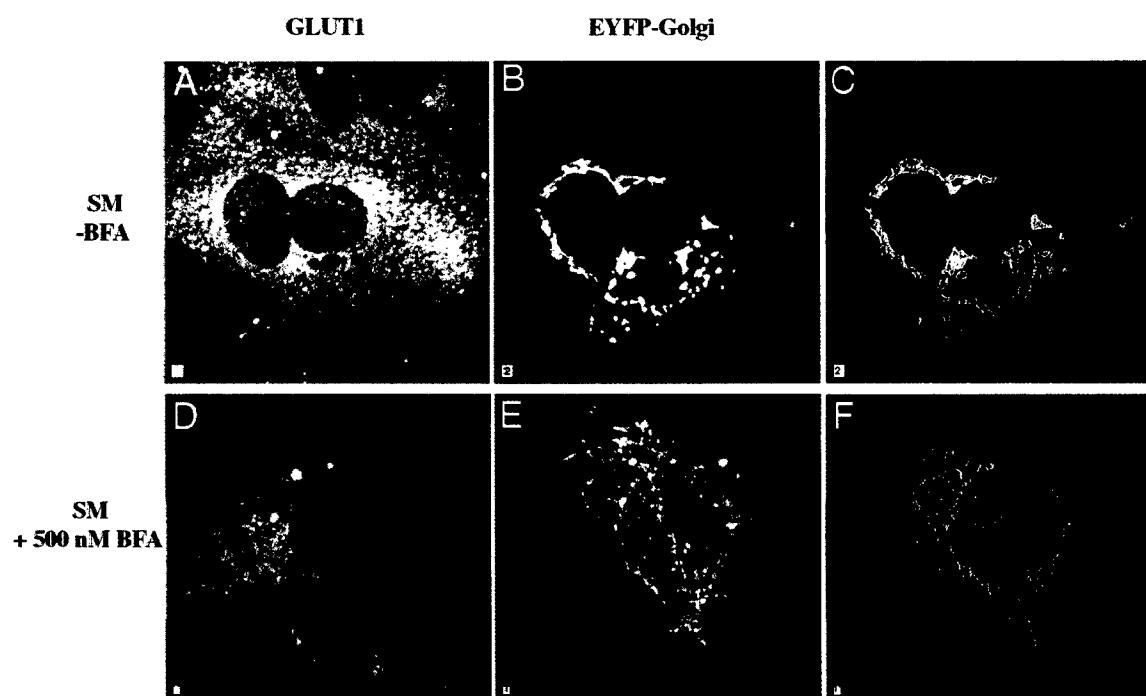


Figure 8

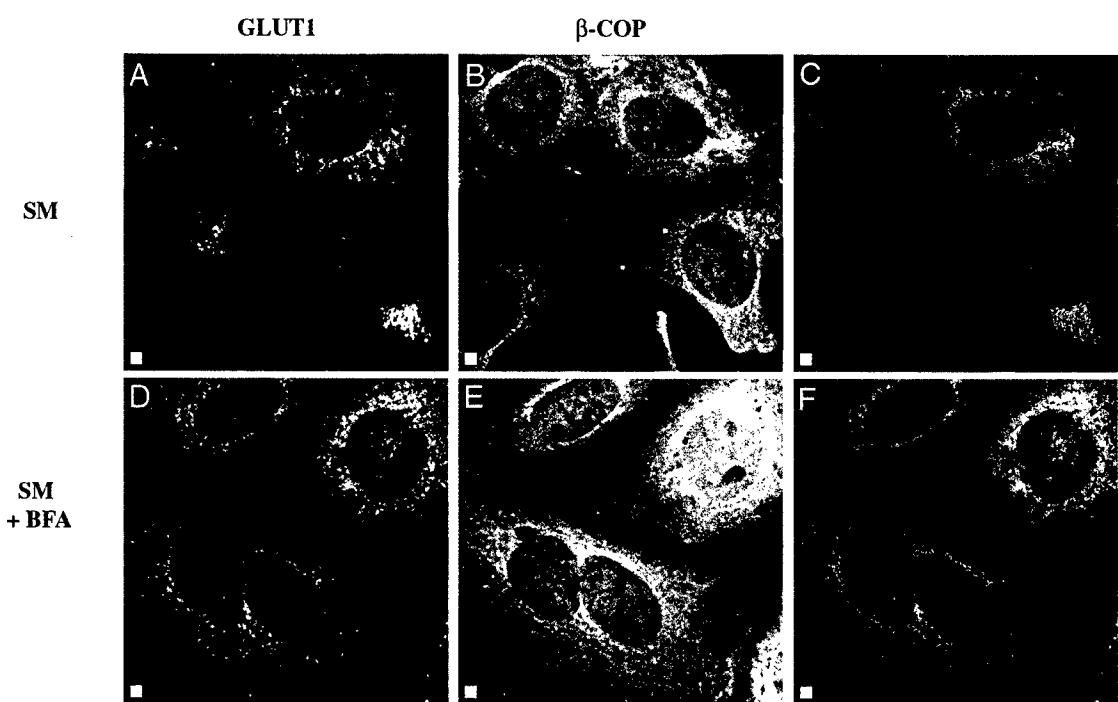
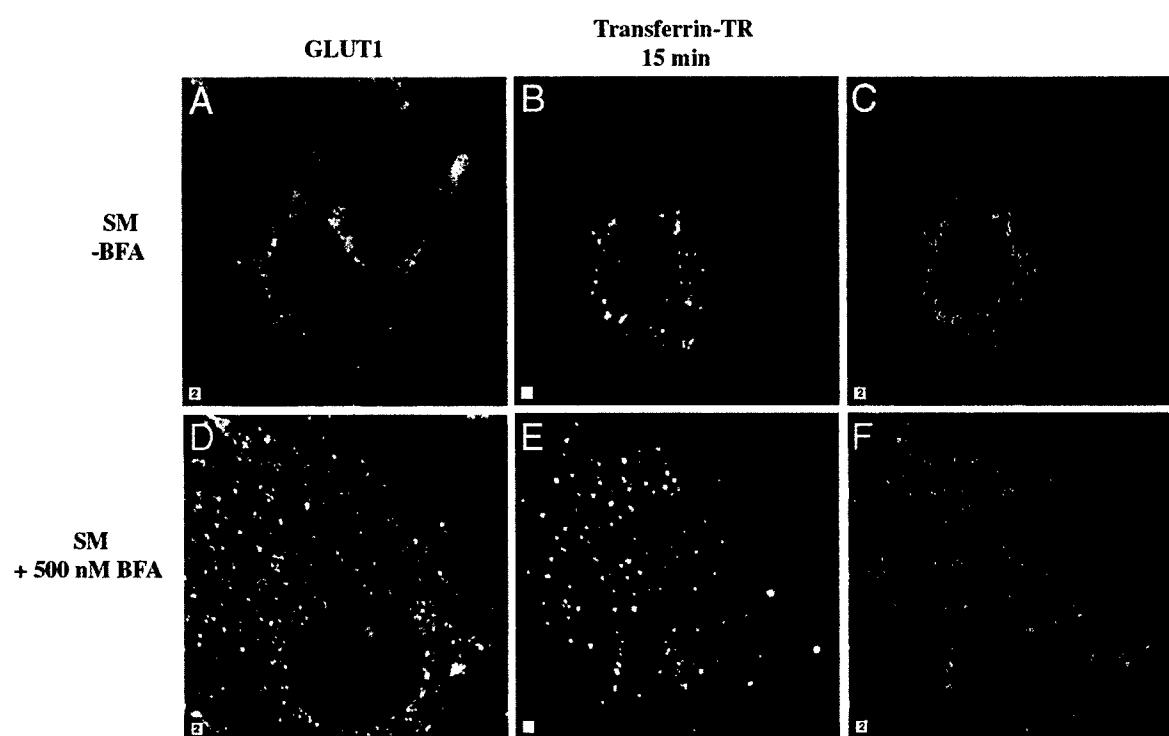


FIGURE 9



Golgi Targeting of the GLUT1 Glucose Transporter in Lactating Mouse Mammary Gland

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ABSTRACT

Lactose, the major carbohydrate of human milk, is synthesized in the Golgi from glucose and UDP-galactose. The lactating mammary gland is unique in its requirement for the transport of glucose into Golgi. Glucose transporter-1 (GLUT1) is the only isoform of the glucose transporter family expressed in mammary gland. In most cells, GLUT1 is localized to the plasma membrane and is responsible for basal glucose uptake; in no other cell type is GLUT1 a Golgi resident. To test the hypothesis that GLUT1 is targeted to Golgi during lactation, the amount and subcellular distribution of GLUT1 were examined in mouse mammary gland at different developmental stages. Methods including immunohistochemistry, immunofluorescence, subcellular fractionation, density gradient centrifugation, and Western blotting yielded consistent results. In virgins, GLUT1 expression was limited to plasma membrane of epithelial cells. In late pregnant mice, GLUT1 expression was increased with targeting primarily to basolateral plasma membrane but also with some

intracellular signal. During lactation, GLUT1 expression was further increased, and targeting to Golgi, demonstrated by colocalization with the 110-kD coatomer-associated protein β -COP, predominated. Removal of pups 18 d after delivery resulted in retargeting of GLUT1 from Golgi to plasma membrane and a decline in total cellular GLUT1 within 3 h. In mice undergoing natural weaning, GLUT1 expression declined. Changes in the amount and targeting of GLUT1 during mammary gland development are consistent with a key role for GLUT1 in supplying substrate for lactose synthesis and milk production. (*Pediatr Res* 47: 444–450, 2000)

Abbreviations

β -COP, 110-kD coatomer-associated protein

GLUT1, glucose transporter-1

PBS/T, PBS, 0.2% Tween 20, pH 7.3

UDP-galactose, uridine-diphosphogalactose

The recommendation by pediatricians that mothers breastfeed (1) is based on the unique superiority of human milk and on the nutritional, neurodevelopmental, immunologic, and psychologic advantages it confers. Yet our understanding of the elaborate machinery responsible for milk synthesis and secretion and the manner of its regulation in health and disease is rudimentary. Advances in knowledge of the cellular physiology of the mammary gland may provide avenues to influence the quantity and quality of milk produced by nursing mothers and increase the rate and average duration of breast-feeding.

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The most common explanation for premature cessation of breast-feeding is the mother's perception that her milk production is inadequate (2, 3). Because lactose is the major carbohydrate and osmotic constituent of human milk, the volume of milk produced is a function of the rate of lactose synthesis. Lactose synthesis takes place within the Golgi (4) and is catalyzed by lactose synthetase, a complex of galactosyltransferase and the mammary gland-specific protein α -lactalbumin. The substrates for lactose synthesis are UDP-galactose and free glucose. Direct measurement of intracellular glucose concentration demonstrated that glucose transport into the mammary epithelial cell may be rate-limiting for lactose synthesis (5). The mammary epithelial cell not only must transport glucose from the blood across the basal membrane into the cell but also must deliver this glucose to the Golgi. The requirement for free glucose within the Golgi is unique to the lactating mammary gland. A Golgi-specific glucose carrier protein accounting for Golgi glucose uptake during lactation was proposed (6) 5 y before the cloning of GLUT1 (7), the first member of the family of facilitated diffusion glucose transporter isoforms.

GLUT1 is the only isoform of the facilitated diffusion family of glucose transporters known to be expressed in mammary gland. GLUT1 is targeted to the plasma membrane in most cell types. No glucose transporter isoform has been proven to target to the Golgi, nor is there evidence of any other mediator of glucose transport into Golgi. Five years after the discovery of GLUT1, its potential role in lactation was examined for the first time. Subcellular fractionation and Western blotting of d-10 lactating rat mammary glands suggested that during lactation, GLUT1 may also be found in the Golgi (8), but no other time points were examined. Because this study relied exclusively upon subcellular fractionation, the possibility that Golgi fractions were contaminated with other organelles cannot be excluded. In other studies (9, 10) in d-10–12 lactating rat mammary gland relying only upon microscopy, high and polarized expression of plasma membrane GLUT1 during lactation was seen, and total cellular GLUT1 content declined after weaning pups for 24 h (9). However, Golgi targeting of GLUT1 was not observed. These contradictory results demonstrated to us the need for a comprehensive multifaceted approach to examine whether or not GLUT1 is a candidate Golgi glucose transporter during lactation.

Therefore, this study examined whether changes in the amount, activity, and subcellular targeting of GLUT1 in lactating mouse mammary gland are regulated in a manner consistent with a possible role for GLUT1 in supplying free glucose to Golgi as a substrate for lactose synthesis. Specifically, we hypothesized that during lactation, the amount of GLUT1 would increase and a transition from plasma membrane targeting to Golgi targeting would occur. Furthermore, we tested the hypothesis that under conditions of forced weaning, abruptly curtailing milk synthesis, Golgi targeting of GLUT1 would diminish. To provide multiple lines of evidence, methods included subcellular fractionation and density gradient centrifugation, epifluorescent and confocal immunofluorescent microscopy, and immunohistochemistry.

METHODS

Antisera and reagents. GLUT1 antibody was a well-characterized highly specific rabbit polyclonal antiserum to human GLUT1 raised against synthetic peptide made up of the 16 C-terminal amino acids, a kind gift of Dr. M. Mueckler (Washington University School of Medicine, St. Louis, MO). This antibody was affinity purified using the same synthetic peptide bound to thiopropyl sepharose (Pharmacia, Piscataway, NJ) (11) before use for immunoblotting or immunocytochemistry. Mouse MAb to rat β -COP were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein-labeled goat anti-rabbit antibodies and Texas-red sheep anti-mouse antibodies were from ICN (Aurora, OH). Reagents were from Sigma Chemical Co. unless otherwise specified.

Animals. Nulliparous female CD-1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were mated, and, at parturition (d 0 of lactation), the litters were adjusted to 10 pups. Animals were fed on Purina lab chow (Ralston Purina, St. Louis, MO) and had access to water *ad libitum* and a daily photoperiod of 12 h. Experiments were carried out in duplicate or triplicate using different animals, and representative results are shown. Studies were reviewed and approved by the Animal Studies Committees of the Washington University School of Medicine and the Baylor College of Medicine.

Subcellular fractionation and density gradient centrifugation. One mammary gland preparation per animal was prepared as follows. Mammary glands were removed and rinsed twice with ice-cold PBS and once with sucrose solution (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, pH 7.8), resuspended in a small volume of homogenization buffer (PBS, 1 mM EDTA), and homogenized with five strokes in a tight-fitting Dounce homogenizer. After centrifugation at 3000 \times *g* for 10 min at 4°C, the supernatant was centrifuged at 17 000 \times *g* for 10 min at 4°C. This supernatant was centrifuged at 100 000 \times *g* for 30 min at 4°C. In certain experiments, the

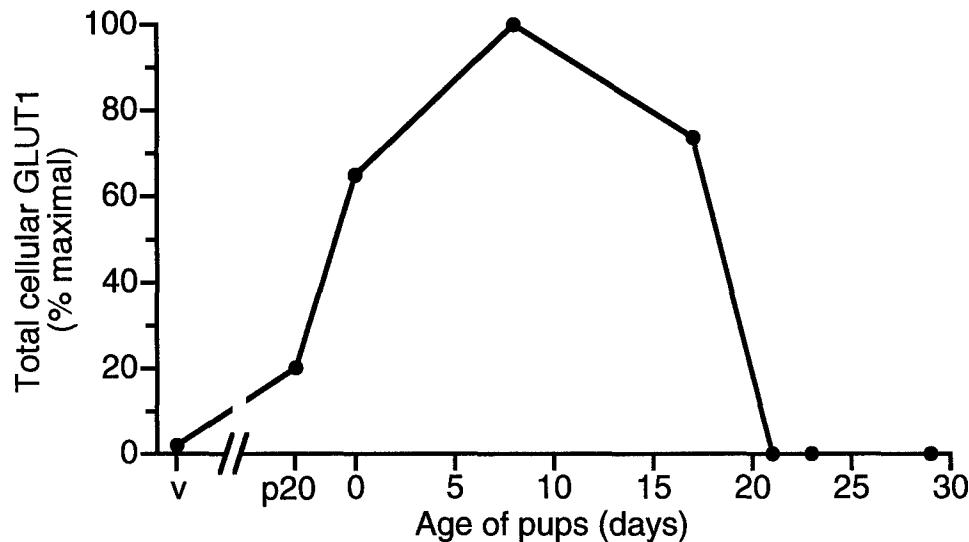


Figure 1. GLUT1 induction during pregnancy and lactation. Homogenate fractions from mouse mammary gland were subjected to SDS-PAGE, Western blotting, enhanced chemiluminescence (ECL), and laser densitometry as described in "Methods." Samples containing 10 μ g of total cellular protein were run on the same gel, and ECL exposure time was 1 min. Results are expressed relative to peak GLUT1 expression, which was observed 8 d after delivery. GLUT1 rises from very low levels in virgin gland during pregnancy, increases further during lactation, then rapidly declines upon weaning.

17 000-g pellet was resuspended and subjected to density gradient centrifugation in a self-generating iodixanol density gradient (10–37%) at 180 000 \times *g* for 3 h at 4°C. Fractions were collected from the top (Labconco Auto-densi Flow, Kansas City, MO), and the lowest density fraction (1.05–1.08 g/cm³) was analyzed. Alkaline phosphatase (12) and galactosyltransferase (13) were assayed as described.

Western blotting. Samples were prepared and subjected to standard SDS-PAGE on 10% gels as previously described (14). Proteins were immobilized on nitrocellulose by wet transfer. Primary antibody was the peptide affinity purified GLUT1 antibody described above (1 μ g/mL in 5% nonfat dry milk in PBS). Horseradish peroxidase-linked donkey anti-rabbit antibody (Amersham, Piscataway, NJ) served as secondary antibody, and signal was detected using ECL-Plus (Amersham). Quantitative differences in signal strength were measured using a laser densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemical staining. Mammary tissue was fixed in 10% neutral buffered formalin supplemented with zinc chloride (Anatech, Ltd., Battle Creek, MI) and processed for paraffin sections by using standard techniques. Tissues were embedded in paraffin and sectioned at 4 μ m on a rotary microtome (Leitz 1512), collected on standard glass microscope slides, and stained with hematoxylin and eosin by using a routine Harris hematoxylin solution and alcoholic eosin counterstain. Sections were affixed to capillary gap glass microscope slides (Ventana Medical Systems, Inc., Tucson, AZ), dried at 60°C for 1 h, and deparaffinized. The tissue sections were incubated for 20 min in a 1:10 citrate buffer solution (Dako Corporation, Carpinteria, CA) in a steam environment to enhance antigen availability and then incubated in the same solution for an additional 20 min at room temperature. The sections were rinsed in PBS/T to enhance flow in the capillary gap; PBS/T was used to rinse the sections between all steps of the protocol. All reagents used in the immunohistochemical procedure were made in PBS/T supplemented with 0.5% crystalline grade BSA (Sigma Chemical Co.) as a protein carrier. GLUT1 immunohistochemistry was performed using a Tek-Mate 500 automated system (Ventana Medical Systems, Inc.). Sections were incubated in a 1:75 solution of normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. The serum was removed, anti-GLUT1 antibody was applied at a concentration of 2.5 μ g/mL, and the sections were incubated in a humid chamber at room temperature overnight. Negative control sections were incubated in diluent rather than the GLUT1 antibody. After incubation, sections were rinsed and a biotin-conjugated goat anti-rabbit IgG (Vector Laboratories) was applied at 2.25 μ g/mL for 45 min at room temperature. This was followed by endogenous peroxidase exhaustion using 3% H₂O₂ in absolute methanol, three changes of 5 min each. The sections were treated with a peroxidase-tagged avidin-biotin complex (Vector Laboratories) for 45 min at room temperature. Antigenic sites were visualized using diaminobenzidine enhanced with 1% nickel chloride as the chromogen (Sigma Chemical Co. Chemicals, St. Louis, MO). The tissue sections were counterstained with eosin, then processed through ascending grades of ethyl alco-

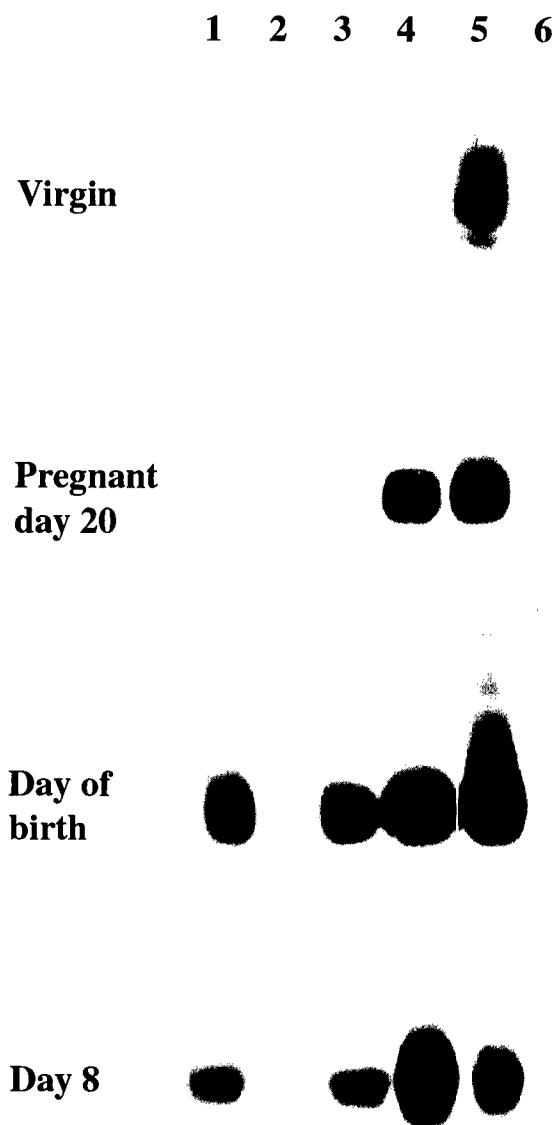


Figure 2. Golgi targeting of GLUT1 during lactation. Subcellular fractionation of mammary gland was carried out as described in "Methods." Lane 1, homogenate; lane 2, 3000-g (nuclear) pellet; lane 3, 3000-g supernatant; lane 4, 17 000-g (Golgi-enriched) pellet; lane 5, 100 000-g (plasma membrane-enriched) pellet; lane 6, 100 000-g supernatant (cytosol). Samples contained 60 μ g of protein except for the virgin samples, which contained 25 μ g. ECL exposure times were adjusted as needed to assess relative targeting to Golgi-enriched and plasma membrane-enriched fractions while avoiding saturation of signal from any one fraction and ranged from overnight for the virgin samples to 30 s for the samples from d 8. Total GLUT1 expression at these time points was directly compared in Figure 1. GLUT1 targeting shifts from predominance in the plasma membrane-enriched fraction in the virgin, to approximately equivalent plasma membrane and Golgi targeting in late pregnancy and on the day of delivery, to predominance in the Golgi-enriched fraction by d 8.

hol and xylene, and mounted on coverslips by use of a synthetic mountant.

Immunofluorescent staining. Sections were prepared as described above. After incubation with primary antibody against GLUT1, sections were rinsed and an FITC-conjugated antibody directed against rabbit IgG (Dako Corporation) was applied at a concentration of 1:30 for 45 min in the dark. Sections were washed well, mounted in a nonfluorescent aque-

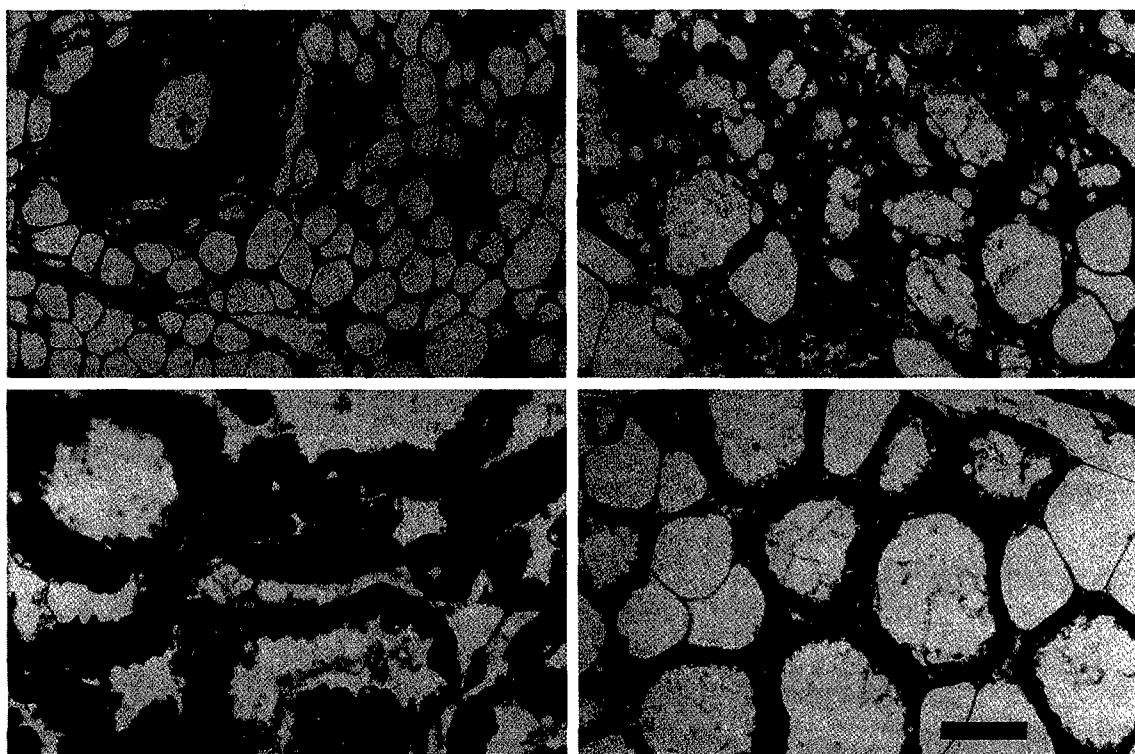


Figure 3. Basolateral plasma membrane targeting of GLUT1 during lactation. (A) virgin, (B) pregnant d 20, (C) lactating (d 18 after delivery), and (D) weaning (d 21 after delivery). Bar, 60 μ m. Immunocytochemistry was carried out as described in "Methods." Positive staining for GLUT1 is indicated by brown. Control slides showed no signal. The virgin gland is predominantly fat, but nonpolarized plasma membrane targeting of GLUT1 is seen in islands of mammary epithelial cells. During lactation, GLUT1 staining is intense but is observed only in basolateral plasma membrane and not in apical plasma membrane.

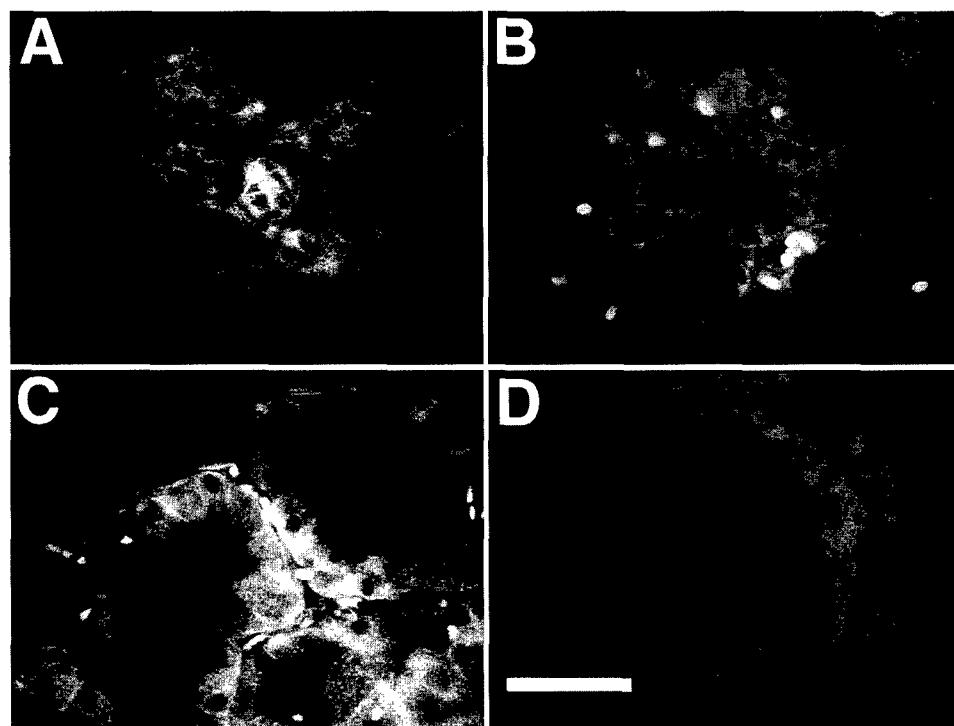


Figure 4. Intracellular targeting of GLUT1 during lactation. (A) virgin, (B) pregnant d 20, (C) lactating (d 18 after delivery), and (D) weaning (d 21 after delivery). Bar, 60 μ m. Immunofluorescent staining and microscopy were carried out as described in "Methods." Control slides showed a level of signal equivalent to that seen in weaning gland. Nonpolarized plasma membrane targeting of GLUT1 in virgin gland and polarized targeting of GLUT1 in the lactating gland are seen. In addition, strong intracellular signal is observed in the lactating but not in the virgin gland. The weaning gland shows only nonspecific staining. Strong signal is observed in red blood cells due to autofluorescence.

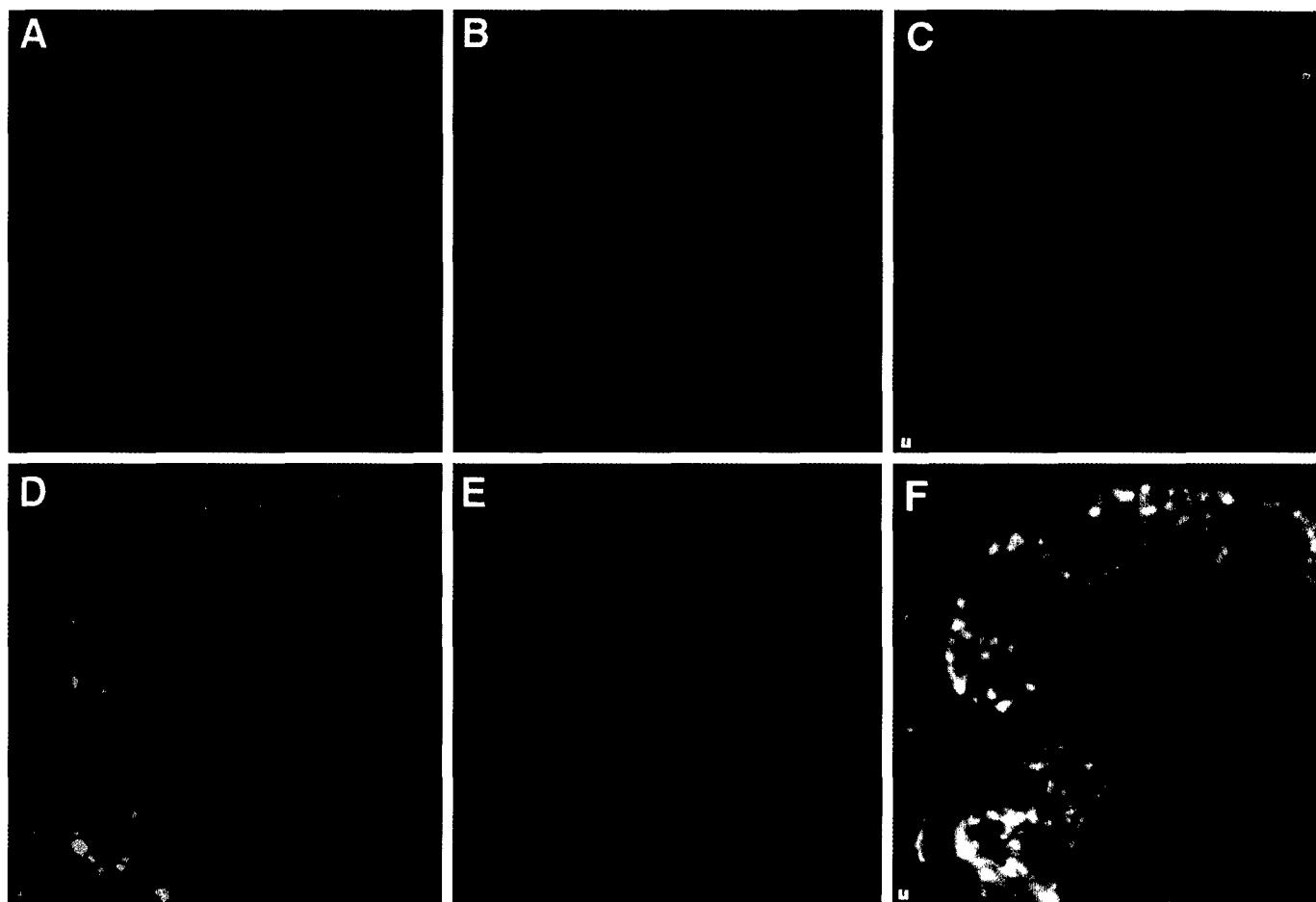


Figure 5. Colocalization of GLUT1 with the Golgi marker β -COP during lactation. (A) control with GLUT1 preimmune serum, (B) control with secondary antibody only, (C) control signals merged, (D) GLUT1 staining, (E) β -COP staining, and (F) GLUT1 and β -COP signals merged with yellow indicating colocalization. Immunofluorescent staining and microscopy were carried out as described in "Methods." Bar, 1 μ m. The high degree of colocalization of GLUT1 with the Golgi marker β -COP (F) demonstrates that GLUT1 is also targeted to Golgi. Control panels A, B, and C demonstrate that the staining shown in D, E, and F is specific.

ous medium, and viewed with a Zeiss Axiophot epifluorescent microscope at a wavelength of 490 nm. The images are shown as equivalent exposures acquired by a Cohu (San Diego, CA) 4910 uncooled charge-coupled device camera; no enhancement or intensification was performed. For confocal microscopy slides, GLUT1 antibody was used as primary antibody as described above, and β -COP antibody was also used as primary antibody at 1:80. Then, fluorescein-labeled goat anti-rabbit antibodies and Texas-red sheep anti-mouse antibodies were both applied as described above. Also, for confocal microscopy, preimmune GLUT1 serum rather than diluent was used as a negative control. A Molecular Dynamics Multiprobe 2010 inverted confocal laser scanning microscope was used.

RESULTS

Developmental changes in GLUT1 expression. GLUT1 expression in the mouse mammary gland (Fig. 1) was studied in virgins, in late pregnancy (d 20), on the day of delivery (d 0), at midlactation (d 8), at the peak of lactation (d 18), and then at different time points during weaning (d 21, 23, and 29). Expression gradually rose from an extremely low level in virgins, increased on the day of delivery to a peak during

lactation, and then declined to very low levels as weaning progressed (Fig. 1).

To demonstrate whether there are changes in the subcellular targeting of GLUT1 as it is induced during pregnancy and lactation, subcellular fractionation was used to prepare a 17 000-g pellet enriched in Golgi and a 100 000-g pellet enriched in plasma membrane. Enrichment was demonstrated by assays of marker enzymes. Relative to total cellular levels of each marker, the activity of galactosyltransferase, a Golgi marker, was enriched 3.5- to 4.3-fold in the Golgi-enriched fraction, whereas alkaline phosphatase, a marker of plasma membrane, was enriched 4.5- to 8.6-fold in the plasma membrane-enriched fraction. Virgins demonstrated predominance of GLUT1 in the plasma membrane-enriched fraction, whereas mammary gland differentiation during pregnancy and lactation was associated with an increase in GLUT1 targeting to the Golgi-enriched fractions (Fig. 2). Quantitation by laser densitometry showed that GLUT1 was preferentially targeted to the Golgi-enriched fraction, as indicated by 3.9-fold enrichment on d 8 (Fig. 2) compared with an enrichment of 1.9-fold in the plasma membrane-enriched fraction. As expected, there was no significant targeting of GLUT1 to the 3000-g pellet, which is

enriched in nuclei, or to the 100 000-g supernatant, which is enriched in cytosol. Immunohistochemistry, a method well suited for evaluation of plasma membrane staining, demonstrated labeling of plasma membrane in mammary gland of virgin, pregnant, and lactating mice (Fig. 3). The increase in GLUT1 expression during pregnancy is partially accounted for by the proliferation of mammary epithelial cells. Staining for GLUT1 was not observed during weaning, consistent with results of Western blotting. Virgin gland demonstrated a predominance of fat cells, but the mammary epithelial cells showed significant plasma membrane targeting to both the basolateral and apical membrane. In contrast, during lactation, there was intense staining of the basolateral membrane and no staining of the apical plasma membrane, indicating a polarization of membrane targeting. Immunofluorescent microscopy, which is suitable for evaluation of intracellular as well as plasma membrane targeting, confirmed nonpolarized targeting of GLUT1 in the virgin gland and an increase in expression during pregnancy and lactation (Fig. 4). In the lactating gland, intracellular staining and basolateral plasma membrane staining were seen. No specific staining was seen in mammary gland during weaning. To further define the subcellular targeting of GLUT1 during lactation, confocal immunofluorescent microscopy, which detects signal in a single plane and is more suitable for definition of intracellular targeting, was used to evaluate d-18 lactating mammary gland stained for GLUT1 and β -COP, a Golgi marker (15). GLUT1 was distributed in a predominantly perinuclear punctate pattern with some vesicular and plasma membrane staining as well (Fig. 5D). β -COP was similarly distributed but with a somewhat more diffuse pattern (Fig. 5E). The yellow signal in Figure 5F results from merging red and green signals in Figure 5, D and E, and the abundant yellow signal indicates a high degree of colocalization of GLUT1 with the Golgi marker β -COP. Figure 5, A–C, demonstrates the insignificance of nonspecific staining.

Reversible changes in GLUT1 content and subcellular targeting during forced weaning. The removal of nursing 18-d-old pups from their dams resulted in changes in the amount and subcellular targeting of mammary gland GLUT1 within 3 h (Fig. 6). A decline in total GLUT1 content and a change from predominance of GLUT1 targeting in the Golgi-enriched fraction to predominance in the plasma membrane-enriched fraction were observed. Iodixanol density gradient centrifugation of the 17 000-g pellet was used to provide further enrichment. Because Golgi membranes have the lowest buoyant density of any subcellular membrane fraction, the lowest density fraction, corresponding to a density of 1.05–1.08 g/cm³, was analyzed. A fall in Golgi enrichment of GLUT1 occurred within 3 h, and there was no Golgi enrichment of GLUT1 by 5 h (Fig. 7). However, when pups were then returned to the dam for 5 h, Golgi enrichment of GLUT1 was again observed. When pups were returned for 15 h, Golgi enrichment of GLUT1 was fully restored.

DISCUSSION

The production of an adequate volume of milk by the nursing mother is a prerequisite for successful lactation. Be-

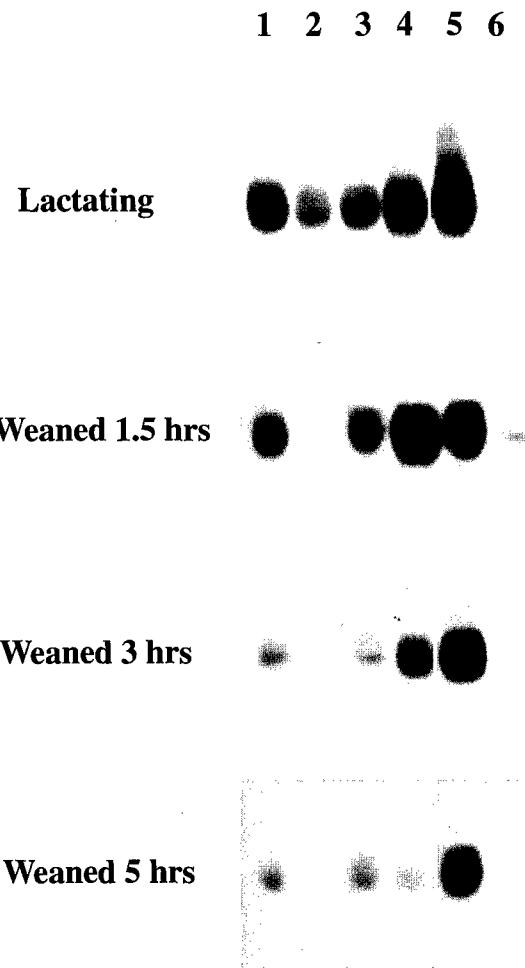


Figure 6. Decline in GLUT1 expression and in Golgi targeting of GLUT1 during forced weaning. Eighteen-day-old pups were removed from lactating dams for the specified time. Subcellular fractionation of mammary gland was carried out as described in "Methods." Lane 1, homogenate; lane 2, 3000-g (nuclear) pellet; lane 3, 3000-g supernatant; lane 4, 17 000-g (Golgi-enriched) pellet; lane 5, 100 000-g (plasma membrane-enriched) pellet; lane 6, 100 000-g supernatant (cytosol). Samples contained 60 μ g of protein, and ECL exposure times were identical. Results shown are representative and at any time point varied 20% or less. Declines in the amount and Golgi targeting of GLUT1 are apparent 3 h after weaning and are pronounced by 5 h after weaning.

cause lactose is the major osmotic constituent of human milk, the synthesis of lactose determines the volume of milk produced. Because changes in lactose synthetase activity do not correlate with changes in milk production (16), the process may be regulated at the level of substrate availability. The lactating mammary gland is unique in its requirement for the transport of free glucose across the Golgi membrane. GLUT1 is the only member of the facilitated diffusion glucose transporter family expressed in mammary gland. Neither GLUT1 nor any other isoform of the glucose transporter family is considered a Golgi resident, although a six-amino acid portion of GLUT4 does confer targeting to the trans-Golgi network and insulin-sensitive translocation to the plasma membrane in fat and muscle cells (17).

Several investigators have studied GLUT1 expression in the lactating mammary gland with conflicting results described above. The purpose of the experiments reported here was to

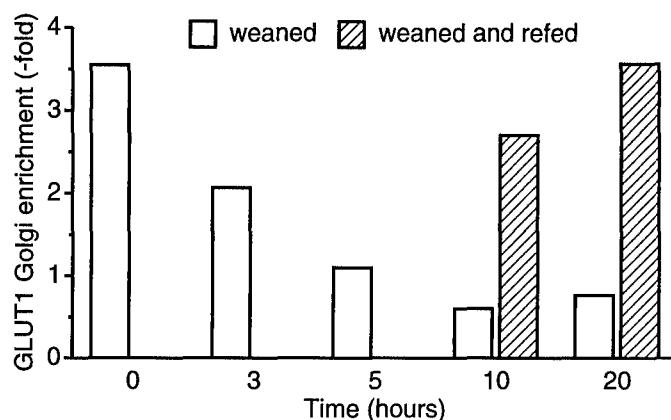


Figure 7. Reversibility of the effect of forced weaning on GLUT1 targeting. Pups were removed from lactating dams for the specified time. In certain experiments, indicated in crosshatch, pups were returned to their dams after a 5-h weaning period. Subcellular fractionation and iodixanol density gradient centrifugation of mammary gland were carried out as described in "Methods." Representative results are expressed as enrichment of the purified Golgi fraction relative to total cellular levels of GLUT1. Golgi targeting of GLUT1 was lost by 5 h and was not observed after 10 or 20 h of weaning. However, when pups were returned to the dam after a 5-h absence, Golgi targeting of GLUT1 was observed within the next 5 h and was fully restored 15 h after lactation was resumed.

systematically test whether the developmental regulation of the amount and subcellular trafficking of GLUT1 in mouse mammary gland indicates an important role for GLUT1 in the provision of substrate for lactose synthesis. In contrast with previous studies, independent methods were used, and multiple time points during the normal developmental cycle and the forced weaning-refeeding cycle were examined.

Importantly, consistent results from independent methods, subcellular fractionation followed by density gradient centrifugation, and epi- and confocal immunofluorescent microscopy indicate that during lactation, GLUT1 is targeted not to the plasma membrane as it is in most cells but to the Golgi. This resolves the contradictory previous studies and leads to the conclusion that GLUT1 is diverted from normal sorting pathways to the Golgi. Preferential targeting of GLUT1 to basolateral compared with apical membrane during lactation is consistent with the need of the mammary epithelial cell to take up glucose from the blood. More work is needed to demonstrate that GLUT1 actually controls the provision of glucose to the Golgi for lactose synthesis. The results do not exclude the previously suggested (8) possibility that a novel transporter, yet to be identified, resides in the Golgi.

The results suggest a tissue- and developmental stage-specific Golgi targeting mechanism for GLUT1. The structural determinants of targeting of proteins to Golgi are controversial. The transmembrane-spanning domain or specific amino acid motifs within it have seemed important (18). However, no common retention signal is apparent on examination of a large number of cloned glycosyltransferases (19). Other proposed explanations include the formation of nonmobile protein oligomers or the influence of the high cholesterol content of Golgi membranes on mobility of resident Golgi enzymes (20). Be-

cause our general understanding of the determinants of protein targeting to Golgi is limited, it is not surprising that the molecular basis of the hormonally regulated Golgi targeting of GLUT1 is unclear. Further study may reveal mechanisms regarding GLUT1 targeting that are relevant to other Golgi proteins as well. Hormonal regulation of GLUT1 subcellular targeting suggests a flexibility of the Golgi targeting machinery that has not previously been appreciated.

The reversible nature of the targeting of GLUT1 to Golgi and the time course over which changes in GLUT1 targeting are observed suggest a dynamic process that requires frequent suckling to maintain substrate supply for lactose synthesis. Future work will explore the relevance of this mechanism to the phenomenon of decreased production of human milk when nursing intervals are prolonged.

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REFERENCES

1. Work Group on Breastfeeding. American Academy of Pediatrics 1997 Breastfeeding and the use of human milk. *Pediatrics* 100:1035-1039
2. Bourgoin GL, Lahaie NR, Rheaume BA, Berger MG, Dovigi CV, Picard LM, Sahai VF 1997 Factors influencing the duration of breastfeeding in the Sudbury region. *Can J Public Health* 88:238-241
3. Essex C, Smale P, Geddis D 1995 Breastfeeding rates in New Zealand in the first 6 months and the reasons for stopping. *NZ Med J* 108:355-357
4. Keenan TW, Morre DJ, Cheetham RD 1970 Lactose synthesis by a Golgi apparatus fraction from rat mammary gland. *Nature* 228:1105-1106
5. Wilde CJ, Kuhn NJ 1981 Lactose synthesis and the utilisation of glucose by rat mammary acini. *Int J Biochem* 13:311-316
6. White MD, Kuhn NJ, Ward S 1980 Permeability of lactating-rat mammary gland Golgi membranes to monosaccharides. *Biochem J* 190:621-624
7. Mueckler M, Caruso C, Baldwin SA, Panico M, Blench J, Morris HR, Allard WJ, Lienhard GE, Lodish HF 1985 Sequence and structure of a human glucose transporter. *Science* 229:941-945
8. Madon RJ, Martin S, Davies A, Fawcett HA, Flint DJ, Baldwin SA 1990 Identification and characterization of glucose transport proteins in plasma membrane- and Golgi vesicle-enriched fractions prepared from lactating rat mammary gland. *Biochem J* 272:99-105
9. Camps M, Vilardo S, Testar X, Palacin M, Zorzano A 1994 High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. *Endocrinology* 134:924-934
10. Takata K, Fujikura K, Suzuki M, Suzuki T, Hirano H 1997 GLUT1 glucose transporter in the lactating mammary gland in the rat. *Acta Histochem Cytochem* 30:623-628
11. Parekh BS, Schwimmbeck PW, Buchmeier MJ 1989 High efficiency immunoaffinity purification of anti-peptide antibodies on thiopropyl sepharose immunoabsorbents. *Pept Res* 2:249-252
12. Langridge-Smith JE, Field M, Dubinsky WP 1998 Isolation of transporting plasma membrane vesicles from bovine tracheal epithelium. *Biochim Biophys Acta* 731:318-328
13. Graham JM 1993 The identification of subcellular fractions from mammalian cells. In: Graham JM, Higgins JA (eds) *Biomembrane Protocols: I. Isolation and Analysis*. Humana Press, Totowa, NJ, pp 1-18
14. Haney PM, Slot JW, Piper RC, James DE, Mueckler M 1991 Intracellular targeting of the insulin-regulatable glucose transporter (GLUT4) is isoform specific and independent of cell type. *J Cell Biol* 114:689-699
15. Duden R, Griffiths G, Frank R, Argos P, Kreis TE 1991 Beta-COP, a 110-kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to beta-adaptin. *Cell* 64:649-665
16. Bushway AA, Park CS, Keenan TW 1979 Effect of pregnancy and lactation on glycosyltransferase activities of rat mammary gland. *Int J Biochem* 10:147-154
17. Haney PM, Levy MA, Strube MS, Mueckler M 1995 Insulin-sensitive targeting of the GLUT4 glucose transporter in L6 myoblasts is conferred by its COOH-terminal cytoplasmic tail. *J Cell Biol* 129:641-658
18. Nilsson T, Warren G 1994 Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr Opin Cell Biol* 6:517-521
19. Keenan TW 1998 Biochemistry of the Golgi apparatus. *Histochem Cell Biol* 109:505-516
20. Munro S 1998 Localization of proteins to the Golgi apparatus. *Trends Cell Biol* 8:11-15

PROLACTIN INDUCES TARGETING OF GLUT1 TO GOLGI IN HUMAN
MAMMARY EPITHELIAL CELLS

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The synthesis of lactose, the major osmotic constituent of milk, determines the volume of milk production in humans. A primary function of the differentiated mammary epithelial cell is the synthesis of lactose, within the Golgi, from UDP-galactose and free glucose. The final step in the biosynthesis of lactose is catalyzed by lactose synthetase, a complex of α -lactalbumin and the Golgi enzyme β 1,4-galactosyltransferase¹. The mammary gland is unique in its requirement for transport of free glucose into the Golgi. GLUT1, the only glucose transporter expressed in mammary epithelial cells²⁻⁴, normally resides in the plasma membrane. Its role in glucose transport into Golgi of rodents has been controversial^{3,5,6}. Human milk contains about three times more lactose than rodent milk. Here we utilize immunofluorescent staining of human mammary epithelial cells to demonstrate prolactin-dependent colocalization of GLUT1 with several Golgi markers. This illustrates a potential mechanism for the delivery of free glucose to the Golgi, in what may be the rate-limiting step for lactose synthesis and milk production. In addition to its widely recognized role in the uptake of glucose by cells, GLUT1 may also mediate glucose transport between intracellular compartments.

Immunofluorescent microscopy of human mammary epithelial cells in maintenance medium using anti-GLUT1 antibody demonstrated primarily plasma membrane distribution of GLUT1, as well as some intracellular staining, mostly in a perinuclear pattern (Fig. 1A). After exposure to prolactin-rich medium for 4 days, GLUT1 was specifically targeted intracellularly, demonstrating a perinuclear punctate pattern as well as apparent nuclear membrane staining. Plasma membrane staining was markedly reduced (Fig. 1B). In cells exposed to prolactin, perinuclear GLUT1 colocalized with ECFP-Golgi, a cyan fluorescent protein fused to a membrane-anchoring signal specific to β 1,4-galactosyltransferase, identifying the *medial/trans* region of the Golgi⁷(Fig. 2A-C). Immunocytochemistry of cells exposed to prolactin also demonstrated colocalization of GLUT1 with α -lactalbumin, the milk whey protein that associates with galactosyltransferase to form lactose synthetase¹(Fig. 2D-F), and with α -mannosidase II, a *medial*-Golgi marker⁸(Fig. 2G-I). GLUT1 also showed partial colocalization with β -COP, a *cis*-Golgi marker that also marks the *trans*-Golgi network and the ER-Golgi boundary⁹(Fig. 2J-L). GLUT1 did not colocalize with BODIPY-TR ceramide, a *trans*-Golgi marker¹⁰(Fig. 2M-O). A brief exposure to transferrin-Texas Red served to mark endosomes¹¹, while prolonged exposure marked lysosomes¹². Some perinuclear punctate staining of GLUT1 corresponded to endosomes (Fig. 2P-R), but not to lysosomes (Fig. 2S-U). Nonspecific signal was negligible under all conditions.

Upon exposure of human mammary epithelial cells to prolactin, GLUT1 is targeted primarily to the *medial*-Golgi, colocalizing with the components of lactose synthetase complex. The initiation of α -lactalbumin synthesis that occurs at parturition is required for the initiation of copious milk production, but is neither the only factor nor the limiting factor controlling lactose synthesis¹³. Our findings support the importance of GLUT1 for transport of glucose into Golgi, and suggest how a substrate required for lactose synthesis is delivered. Apparent nuclear membrane staining for GLUT1 seen in human mammary epithelial cells has not been reported previously in any cell type, and its significance is a matter for speculation and further study. The suggestion that GLUT1 does not solely act at the plasma membrane, but may function in an intracellular organelle as well, conceptually complements the well-known insulin-regulated targeting of GLUT4¹⁴, and to a lesser extent of GLUT1¹⁵, to their site of action, the plasma membrane, in fat and muscle cells. Our results indicate the existence of a prolactin-induced, cell type-specific, developmental stage-specific sorting machinery for GLUT1 in mammary epithelial cells. The identification of GLUT1 in endosomes suggest that GLUT1 sorting is a continuous, dynamic process. Further work delineating the molecular mechanism of GLUT1 sorting and the targeting determinants it recognizes should improve our understanding of a key regulatory step of milk production in the nursing mother.

METHODS

Cell culture: Human mammary epithelial cells (Clonetics, BioWhittaker, Walkersville, MD) from normal breast tissue biopsies were maintained in medium containing 10 ng/ml hEGF (human recombinant Epidermal Growth Factor), 5 mg/ml insulin and 0.5 mg/ml hydrocortisone. The cells were treated for 4 days in medium containing also 0.5 mg/ml BPE (Bovine Pituitary Extract, Clonetics), which is rich in prolactin.

Transfection with pECFP-Golgi: Liposome-mediated transfection using LipoFectAmine Plus Reagent (GibcoBRL, Life Technologies Inc., Rockville, MD) was performed in 35 mm dishes, containing 5×10^5 cells per plate. Each plate was transfected with 1 μ g of pECFP-Golgi (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Transient transfections were checked for fluorescent signal within 48-60 hours from transfection.

Immunofluorescent staining: Cells were grown on glass coverslips, fixed, and permeabilized with 0.1% Triton X-100 in PBS. Treatment with primary antibody in 0.1% horse serum in PBS was performed overnight at 4°C. Primary antibodies included: (1) peptide-affinity purified mouse polyclonal antibody against the C-terminus of GLUT1¹⁵, 6 μ g/ml; (2) mouse monoclonal antibody against human α -lactalbumin (Clone F20.16) (NeoMarkers, Lab Vision Corp., Fremont, CA), 1:100; (3) mouse monoclonal antibody against rat mannosidase II (Clone 53FC3) (BabCO, Berkeley Antibody Company, Richmond, CA), 1:100; (4) mouse monoclonal antibody against synthetic peptide D1 of β -COP (Clone maD) (Sigma, St. Louis, MO), 1:80. FITC-conjugated goat anti-rabbit antibody and Texas Red conjugated rabbit anti-mouse antibody (diluted 1:100 with 0.1% horse serum in PBS) were used as secondary antibodies. Coverslips were then mounted in

Pro Long anti-fade medium (Molecular Probes, Eugene, OR) on glass slides for microscopic examination.

Staining with BODIPY-TR-ceramide: Cells were grown on glass coverslips and fixed.

Incubation with 5 nmol/ml BODIPY-TR-ceramide (Molecular Probes, Inc. Eugene, OR) was performed on permeabilized cells in the same manner used for the treatment with the primary antibodies, as described above.

Staining with transferrin-Texas Red: Cells were grown on glass coverslips, and incubated with 100 µg/ml of transferrin-Texas Red (Molecular Probes, Inc. Eugene, OR) at 37°C for 15 minutes to mark endosomes, or overnight to mark lysosomes, before fixation, permeabilization and staining with anti-GLUT1 antibody.

Microscopy: Fluorescent signal was detected using an OLYMPUS iX-70 microscope.

Images were captured by a color CCD camera (Optronics, DEI-750 CE Digital Output Model S60675). Exposure was adjusted in a linear manner and separate colour channels were merged as indicated using Adobe Photoshop 5.0.

REFERENCES

1. Strous, G.J. *CRC Crit Rev Biochem* 21, 119-51 (1986).
2. Burnol, A.F., Leturque, A., Loizeau, M., Postic, C. & Girard, J. *Biochem J* 270, 277-9 (1990).
3. Camps, M., Vilaro, S., Testar, X., Palacin, M. & Zorzano, A. *Endocrinology* 134, 924-34 (1994).
4. Shennan, D.B. *J Mammary Biol. Neoplasia*. 3, 247-258. (1998).
5. Madon, R.J. *et al. Biochem J* 272, 99-105 (1990).
6. Takata, K., Fujikura, K., Suzuki, M., Suzuki, T. & Hirano, H. *Acta Histochem Cytochem* 30, 623-8 (1997).
7. Watzele, G., Bachofner, R. & Berger, E.G. *Eur J Cell Biol* 56, 451-8 (1991).
8. Baron, M.D. & Garoff, H. *J Biol Chem* 265, 19928-31 (1990).
9. Griffiths, G., Pepperkok, R., Locker, J.K. & Kreis, T.E. *J Cell Sci* 108, 2839-56 (1995).
10. Pagano, R.E., Sepanski, M.A. & Martin, O.C. *J Cell Biol* 109, 2067-79 (1989).
11. Hopkins, C.R., Gibson, A., Shipman, M. & Miller, K. *Nature* 346, 335-9 (1990).
12. Mayor, S., Presley, J.F. & Maxfield, F.R. *J Cell Biol* 121, 1257-69 (1993).
13. Kuhn, N.J., Carrick, D.T. & Wilde, C.J. *J Dairy Sci* 63, 328-36 (1980).
14. Holman, G.D. *et al. J Biol Chem* 265, 18172-9 (1990).
15. Gould, G.W. *et al. J Biol Chem* 264, 2180-4 (1989).

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FIGURE LEGENDS

Figure 1: Exposure to prolactin causes intracellular targeting of GLUT1. Cells were fixed and exposed to specific anti-GLUT1 primary antibody. Bar, 15 μ m. A. In maintenance medium, GLUT1 demonstrates primarily a plasma membrane distribution as well as some intracellular mostly perinuclear, staining. B. After exposure to prolactin-rich medium for 4 days, GLUT1 was specifically targeted intracellularly, demonstrating a perinuclear punctate pattern, as well as a distinct nuclear membrane staining.

Figure 2: After exposure to prolactin, GLUT1 colocalizes with α -lactalbumin and α -mannosidase II, and partially with ECFP-Golgi, β -COP and endosomes, but does not colocalize with BODIPY-TR-ceramide or lysosomes. Fluorescent images were captured 60 hours after transfection with 1 μ g of pECFP-Golgi. Cells were maintained in prolactin-rich medium for 4 days, before they were fixed and stained with specific anti-GLUT1, anti- α -lactalbumin, anti- α -mannosidase II or anti- β -COP primary antibodies, or stained with BODIPY-TR-ceramide. Some cells were exposed shortly or overnight to transferrin-Texas Red staining before fixation and exposure to anti-GLUT1. GLUT1 is shown in green, and α -lactalbumin, α -mannosidase II or β -COP in red after staining with FITC-conjugated and Texas Red conjugated secondary antibodies, respectively. ECFP-Golgi emits cyan-blue fluorescence when exposed to fluorescent light at the appropriate wavelength. BODIPY-TR-ceramide and transferrin stains appear in red. Bar, 10 μ m. A, D, G, J, M, P, S. GLUT1 signal. B. ECFP-Golgi signal. E, H, K, N, Q, T. α -lactalbumin, α -mannosidase II, β -COP, BODIPY-TR-ceramide and transferrin (short-term and long-

term) signals, respectively. C, F, I, L, O, R, U. Superimposed images. Perinuclear colocalization of GLUT1 and ECFP-Golgi is shown as areas of coincident staining (C). Colocalization of GLUT1 and α -lactalbumin, α -mannosidase II, β -COP, and endosomes appear as areas of coincident staining, giving rise to yellow signal (F, I, L, R). There is little overlap of GLUT1 green signal with BODIPY-TR-ceramide or transferrin signals (O, U).

Figure 1

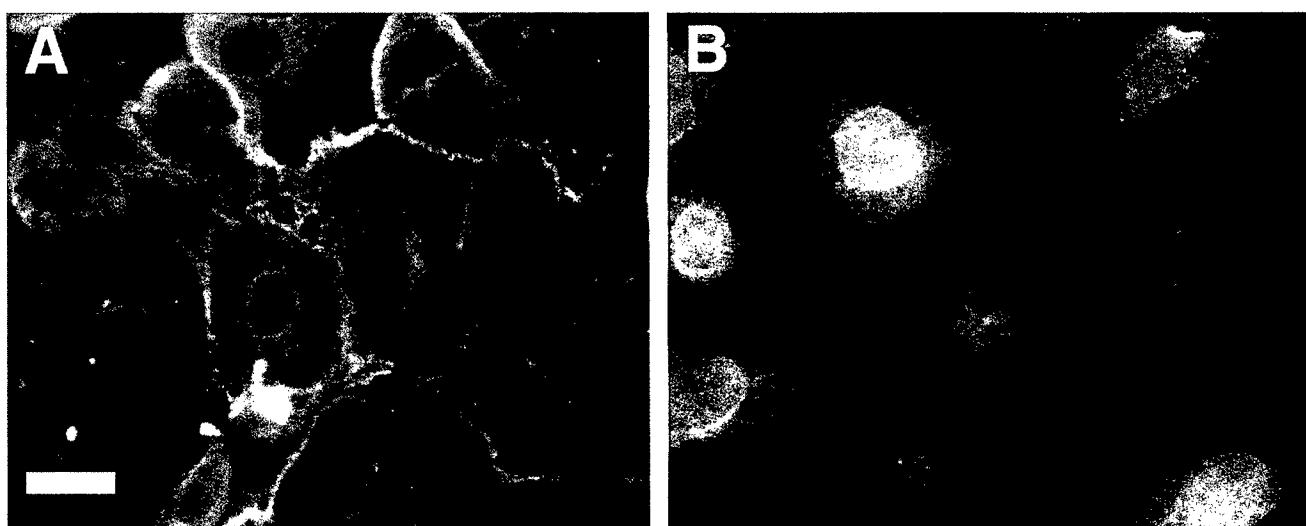
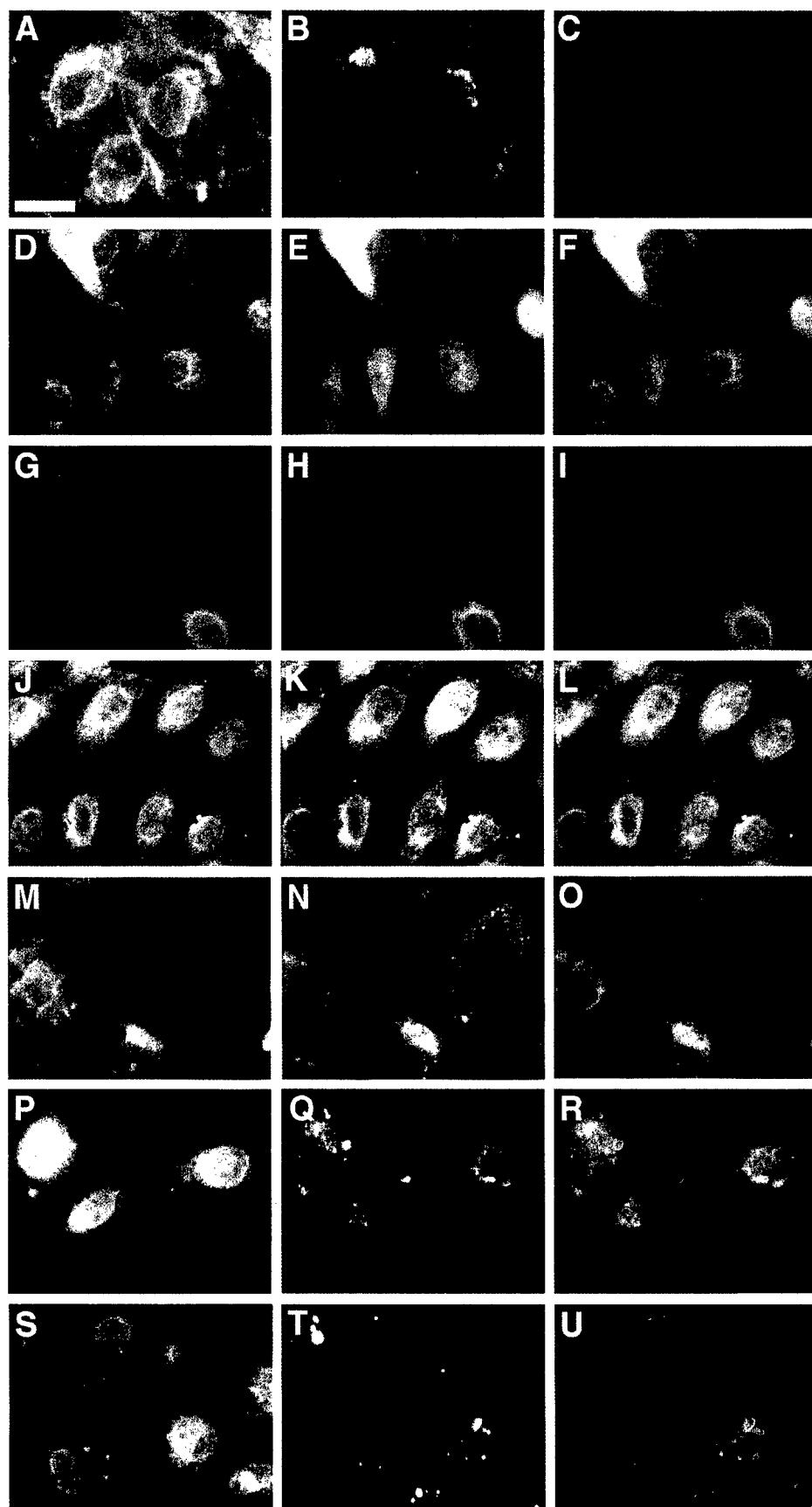


Figure 2



**USEFULNESS OF BLUE, GREEN AND RED FLUORESCENT PROTEIN CHIMERAS
IN THE STUDY OF GLUT1 GLUCOSE TRANSPORTER INTRACELLULAR
TARGETING IN MOUSE MAMMARY EPITHELIAL CELLS**

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Key words: Blue, Green and Red Fluorescent Proteins, GLUT1 glucose transporter, Mammary epithelial cells, Golgi apparatus.

ABSTRACT

GLUT1 is the only glucose transporter isoform expressed in the mammary gland. Hormonally regulated subcellular targeting of GLUT1 from the plasma membrane to Golgi is important for lactose synthesis in lactation. The purpose of this study was to generate mammary epithelial cells expressing blue, green or red fluorescent protein chimeras of GLUT1 in order to assess their usefulness in the study of GLUT1 intracellular targeting. GLUT1 cDNA was subcloned into pEBFP-N1, pEGFP-N1 and DsRed1-N1 (Clontech). EGFP and EBFP are enhanced green and blue variants of the well studies green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and DsRed1 represents a red fluorescent protein from the anemone relative *Discosoma striata*. After liposome-mediated transfection of CIT₃ mouse mammary epithelial cells, fluorescent signal was observed in transient transfections. GLUT1 redistribution from plasma membrane to Golgi in mammary epithelial cells upon conditions mimicking lactation was demonstrated using GLUT1-EGFP chimeras. As opposed to the easily detected green fluorescent signal of these chimeras, the blue fluorescent signal of GLUT1-EBFP fusion chimeras was very weak, and susceptible to rapid photobleaching, thus limiting their usefulness. The newly reported red fluorescent protein DsRed1 has an easily detected red signal, but did not exhibit the expected intracellular pattern seen with EGFP and EBFP. The GLUT1-DsRed1 chimera did not follow the distribution of native GLUT1, nor did it co-localize with GLUT1-EGFP chimera. Thus, chimeras including this red fluorescent protein were not useful in the study of GLUT1 trafficking. Our findings raise major concerns, whether DsRed1 will be useful in the study of other proteins in different cell types. Our future studies will utilize chimeras of GLUT1 and EGFP, rather than EBFP or DsRed1, for co-localization and intracellular trafficking.

INTRODUCTION

The mammary gland is unique in its requirement for transport of free glucose into the Golgi, which is the site of lactose synthesis (6, 25, 29). GLUT1 is the only known isoform of glucose transporter expressed in normal mammary gland (1, 2, 24, 31, 32). It normally resides in the plasma membrane, and its role in glucose transport into Golgi of rodents has been controversial (2, 18, 26). Hormonally regulated subcellular targeting of GLUT1 may have an important role for lactose synthesis in the Golgi of the mammary epithelial cell during lactation (21).

The use of green fluorescent protein (GFP) and its variants as intracellular markers has enhanced the study of intracellular trafficking, and can be applied to analyze glucose transporter targeting.

GFP from the jellyfish *Aequorea victoria* is a reporter molecule for monitoring gene expression and protein localization *in vivo*, *in situ* and in real time (3, 7, 8, 15, 17, 23, 28). GFP is expressed in eukaryotic cells as a fusion protein that serves as a “fluorescent tag”. The wild-type GFP (wtGFP) has a maximum peak of absorbance at 395nm and a minor peak at 470nm, and emits green light maximally at 509nm, with a shoulder at 540nm (13, 28).

EBFP (Enhanced Blue Fluorescent Protein) is a GFP variant with 4 amino acid substitutions that allow the protein to emit blue light. The Tyr-66 to His substitution gives EBFP fluorescence excitation and emission maxima of 380 nm and 440 nm, respectively (12, 22, 30). The other 3 substitutions (Phe-64 to Leu, Ser-65 to Thr and Tyr-145 to Phe) are intended to enhance the brightness and solubility of the protein by improving protein folding properties and efficiency of chromophore formation. Blue Fluorescent Protein (BFP) was initially chosen as the

fusion fluorescent ‘tag’ for GLUT1 to allow future studies of multiple color co-localization studies using green and red fluorescent dyes.

EGFP (Enhanced GFP) carries a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells. It has excitation maximum of 488 nm and emission maximum of 507 nm. EGFP contains two amino acid substitutions (Phe-64 to Leu and Ser-65 to Thr) (5, 22, 30). Its usefulness as a fluorescent ‘tag’ in dynamic intracellular trafficking and targeting studies was reported (9, 14, 16).

DsRed is a newly discovered red fluorescent protein isolated from the IndoPacific sea anemone relative *Discosoma striata* (19). DsRed has maximum excitation and emission at 558 nm and 583 nm, respectively, which is clearly distinct from all the variants of GFP from *Aequorea victoria*, including EBFP and EGFP.

MATERIALS AND METHODS

CIT₃ cells are a non-neoplastic cell line derived from mouse mammary epithelial cells (after being selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions and exhibit polarized transport) (27). CIT₃ cells were provided by M.C. Neville, PhD., University of Colorado School of Medicine. Cells were maintained in growth medium (GM), which is a nutrient-defined basal medium (DMEM/F12), containing 10 μ g/ml insulin and 5ng/ml EGF. To stimulate milk synthesis and secretion, the media was changed to secretion medium (SM), by adding prolactin 3 μ g/ml and hydrocortisone 3 μ g/ml, and withdrawing EGF.

pEBFP-N1 blue fluorescent protein plasmid vectors, pEGFP-N1 green fluorescent protein plasmid vectors, and pDsRed1-N1 red fluorescent protein plasmid vector (# 6069-1, #6085-1 and #6921-1, respectively, Clontech Laboratories Inc., Palo Alto, CA) were used. All the vectors have the human CMV immediate early promoter for high level expression. To ensure maximal mammalian expression, the coding regions of pEBFP, pEGFP and pDsRed1 contain more than 190 silent base mutations, which correspond to human codon-usage preferences (11).

GLUT1 cDNA (20) recovered from pHepG2 using PCR was subcloned into pEBFP-N1, pEGFP-N1 and pDsRed1-N1 respectively, to create C-terminus fusion of GLUT1 to blue, green or red fluorescent proteins. All the vectors were sequenced to verify the right orientation and exclude mutations.

Liposome-mediated transfection of using LipoFectAmine Plus Reagent (#10964013, GibcoBRL, Life Technologies Inc., Rockville, MD) was performed in 35-mm dishes, containing 5 X 10⁵ cells per plate (60-80% confluent). 1 μ g DNA was diluted in basal serum-free medium, DMEM/F12, and 6 μ l of premixed Plus reagent was added to give final volume of 100 μ l. The mixture was incubated at room temperature for 15 minutes to allow DNA-Plus complexes to

form. Cationic liposomes (LipoFectAmine) transfection reagent (4 μ l diluted in 100 μ l DMEM/F12) was combined with the diluted DNA-Plus mixture, and incubated for 15 minutes in room temperature to allow DNA-Plus-liposome complexes to form. While complexes were forming, the medium was replaced with 800 μ l of GM / SM without antibiotics. 200 μ l of the DNA-Plus-liposome complexes solution was added to this medium and gently mixed into it. Cells were incubated in this transfection medium for 3 hours in 37°C in a CO₂ incubator, before 1 ml of full GM / SM was added to the plate. Transient transfections were checked for fluorescent signal at 24-96 hours. Transfections of the vectors only (pEBFP-N1, pEGFP-N1 and pDsRed1-N1) served as controls of gene expression and fluorescent signal detection for the transfections with GLUT1 constructs.

For immunocytochemistry, cells were grown on glass coverslips and fixed. Cells were permeabilized with 0.1% Triton X-100 in PBS. Treatment with primary antibody in 0.1% horse serum in PBS was performed overnight at 4°C. Peptide-affinity purified mouse polyclonal antibody against the C-terminus of GLUT1 (10), 6 μ g/ml, were used as primary antibodies. FITC-conjugated goat anti-rabbit antibody and Texas Red conjugated rabbit anti-mouse antibody (diluted 1:100 with 0.1% horse serum in PBS) were used as secondary antibodies. Coverslips were then mounted in Pro Long anti-fade medium (Molecular Probes, Eugene, OR) on glass slides for microscopic examination.

Fluorescent signal was detected using OLYMPUS iX-70 microscope. Images were captured by an uncooled color CCD camera (Optronics, DEI-750 CE Digital Output Model S60675). Exposure was adjusted in a linear manner and separate colour channels were merged as indicated using Adobe Photoshop 5.0.

RESULTS

EBFP gave a faint blue fluorescent signal, susceptible to photobleaching and requiring enhancement (Fig 1 - A, D). The short exposure time used for the low power image did not reflect strong signal, but was necessary in order to significantly decrease the high background fluorescence, which obscured the signal of the transfected cells. EGFP gave a bright signal with good details, without any need for enhancement (Fig 1 – B, E). EGFP and EBFP showed consistent fluorescent signal distribution throughout the cell with exclusion only of the nucleoli (Fig 1 – D, E; Fig 2 - A).

DsRed1 gave a very bright signal with sharp definition, without any need for enhancement, but its intracellular distribution was inconsistent (Fig 1 – C, F; Fig 2 – B). Native DsRed1 protein did not show the typical distribution throughout the cell with exclusion only of the nucleoli, as GFP variants did (Fig 1 – C; Fig 2 – B). Some cells contained red vesicles, while in others the red signal was distributed evenly throughout the cell. DsRed1 did not co-localize with EGFP (Fig 2 – C).

Only 1-5% of cells transiently transfected with pGLUT1-EBFP displayed detectable blue fluorescent signal, as opposed to 20-30% of the cells that expressed the green signal of GLUT1-EGFP. This does not imply higher transfection efficiency, but rather that the green signal is brighter and easily detectable. Maximal expression of fluorescent signal was at 48-72 hours after transfection for both the blue and the green fusion chimeras (data not shown).

The blue fluorescent signal of EBFP and its fusion chimeras in living CIT₃ cells was weak compared to EGFP green fluorescent signal, and required enhancement in software (Figures 3). The C-terminus fusion of EBFP to GLUT1 was targeted intracellularly, but different patterns of GLUT1 targeting in GM or SM could not be reliably demonstrated. The weakness of

the blue signal also excluded further co-localization studies required in order to define subcellular targeting of GLUT1-EBFP and to follow GLUT1 subcellular trafficking under different conditions.

The high resolution images with the green fluorescent signal of EGFP allowed demonstration of the change in GLUT1 targeting from plasma membrane pattern in GM to intracellular perinuclear distribution, with punctate pattern scattered through the cytoplasm in SM (Fig 4 – A,D). This pattern is consistent with Golgi distribution. The green fluorescent signal of GLUT1-EGFP co-localized with the red signal of native GLUT1 (Fig 5 – A,B,C), demonstrating that the GFP moiety did not alter GLUT1 targeting.

GLUT1-DsRed1 did not demonstrate a consistent pattern in all cells. Although some cells showed red fluorescent patterns consistent with the expected distribution in SM, many others contained the same large red intracellular vesicles, seen also in part of the cells transfected with native DsRed1. None demonstrated the expected plasma membrane pattern in GM (Fig 4 – B, E). GLUT1-DsRed1 red signal did not co-localize with GLUT1-EGFP chimera (Fig 5 – C, F), nor did it co-localize with native GLUT1 (Fig 5 – D,E,F).

DISCUSSION

The purpose of this study was to assess the usefulness of blue, green and red fluorescent protein chimeras to study GLUT1 intracellular targeting in mammary epithelial cells, under different conditions mimicking the change from quiescent state to lactation.

Blue fluorescent protein was the obvious choice initially, since most of the available fluorescent dyes for co-localization studies are in red or green that will not overlap the blue signal of GLUT1. The new EBFP variant (30) reportedly exhibits improved fluorescence with higher level of expression in mammalian cells. This could not be demonstrated in our studies. Not only GLUT1-EBFP chimeras (Fig 3), but also EBFP alone (Fig.1 – A, D) gave a faint blue fluorescent signal susceptible to photobleaching. EBFP and GLUT1-EBFP chimeras did exhibit the expected biological targeting. EBFP alone showed, as expected for GFP variants (7), that the fluorescent signal was distributed throughout the cell with exclusion only of the nucleoli (Fig 1 - D). Blue fluorescent protein chimeras of GLUT1 showed intracellular targeting, but were of limited practical use, because the signal to noise ratio was so low, that it could not have been fully studied even after enhancement. Also, it was not possible to identify different patterns of GLUT1 distribution. No plasma membrane signal was visible in GM, and the intracellular signal was usually recorded only when the cells were fully differentiated, thus limiting the possibility of studying hormonal regulation of GLUT1 intracellular targeting, upon exposure to prolactin in SM.

Comparison of EGFP and EBFP signals (Fig 1 – A, B, D, E), as well as GLUT1 chimeras with EBFP (Fig 3) and with EGFP (Fig 4 – A, D) definitely proved the advantages of EGFP, which gave a bright signal with sharp definition, without any need for enhancement. The weak blue fluorescent signal of EBFP-GLUT1 fusion chimera limits its usefulness in studies of

trafficking kinetics, which require continuous capturing of images over relatively short time courses. Our findings using EBFP, in comparison to EGFP, support previous work by Patterson et al. (22), who studied the physical properties of different mutants of GFP. Most of their data is in comparison to wild type GFP, but can still be useful in respect to our results. They found that EBFP has relatively low quantum yield (17%), and is more susceptible to photobleaching (twofold faster compared to other variants) when excited at 395-nm. They also found that EGFP's high extinction coefficient ($53 \pm 4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) makes its green signal very bright when excited at 488-nm, and thus very useful for microscopic studies. The decreased photostability with the low quantum yield make EBFP more difficult to use for near-UV excitation compared to other green GFP variants. They suggested a role for it as a second label in conjunction with green GFP mutant, such as EGFP. We confirm their conclusions in a practical context. We show that the weak blue signal of EBFP that quickly photobleaches is a problem for imaging, especially when multiple images are taken in kinetic studies. On the other hand, EGFP green signal is strong, and thus very useful for microscopic studies. These properties limit the usefulness of EBFP protein chimeras for intracellular targeting and trafficking studies of GLUT1, and make EGFP the better choice for this purpose. Thus, chimeras of GLUT1 and EGFP, rather than EBFP, will be used for further co-localization and intracellular trafficking studies, under different conditions mimicking quiescent and lactating states.

EGFP showed, as expected (7), that the fluorescent signal was distributed throughout the cell with exclusion only of the nucleoli (Fig 1 – E; 2 -A). GLUT1 intracellular targeting to Golgi in differentiated mammary epithelial cells under hormonal stimulation was demonstrated using GLUT1-green fluorescent protein chimeras. Both the N-terminus and the C-terminus fusions to GLUT1 were targeted from their plasma membrane distribution in GM, to a Golgi related

compartment in SM, demonstrating the typical microscopic perinuclear distribution with a punctate pattern scattered through the cytoplasm (Fig 4 – A, D). This pattern was consistent with the distribution of native GLUT1 (Fig 5 - C). Further co-localization studies to define GLUT1 intracellular targeting are not reported here (unpublished data).

Recently, the new red fluorescent protein (DsRed1) was described (19). As opposed to EBFP and EGFP, which are variants of wild type GFP from *Aequorea victoria*, DsRed1 represents a new group of fluorescent proteins originating from *Discosoma striata*, and naturally emitting in the red wavelength range. Many markers of intracellular compartments (Golgi, endoplasmic reticulum etc.) became available recently for co-localization studies in living cells, but were all attached to GFP variants (e.g., ECFP-Golgi, EYFP-Golgi, EYFP-ER from Clontech Laboratories Inc., Palo Alto, CA), which overlap the emission spectrum we use already to detect our GLUT1-EGFP chimeras. Thus, the possibility of creating red fluorescent protein chimeras of GLUT1 was attractive. However the use of DsRed1 as a fluorescent ‘tag’ to other proteins was problematic. The advantage of GFP and its variants is their ability to serve as ‘fluorescent tag’ to the proteins of interest attached to them, in living cells, without changing their biological activity or localization within the cell (4, 7, 8, 17, 23). The native DsRed1 protein did not consistently show the typical GFP distribution throughout the cell with exclusion only of the nucleoli (7), as do native GFP and its variants (Fig 1 – F; 2 - B in comparison to Fig 1 – D, E; 2 - A). GLUT1-DsRed1 chimera did not show the expected distribution in GM or SM (Fig 4 – B, E), demonstrating inconsistent targeting from cell to cell. It did not co-localize with GLUT1-EGFP chimera (Fig 4 – C, F) or native GLUT1 (Fig 5 - F). Thus, there is no place for this red fluorescent protein in the study of GLUT1 trafficking in mammary epithelial cells.

Our results raise major concerns whether DsRed1 can be used at all for protein targeting and trafficking kinetic studies. Investigators should not assume that DsRed1 chimeras will demonstrate appropriate targeting.

LEGENDS

Figures 1 - CIT₃ cells expressing EBFP, EGFP and DsRed1 fluorescent signals: A, B, C – Low power (10x) images. D, E, F - High power (40x) images. A, D – Blue fluorescent signal of EBFP at 1/60 and ½ sec. respectively. B, E - Green fluorescent signal of EGFP at 1/8 and 1/30 sec. respectively. C, F - Red fluorescent signal of DsRed1 at 1/15 and 1/4 sec. respectively.

Figure 2 - CIT₃ cell expressing EGFP and DsRed1: Images captured 48-60 hours after co-transfection with pEGFP and pDsRed1, at x magnification. A – Green fluorescent signal of EGFP at sec. B – Red fluorescent signal of DsRed1 at sec. C – Combined image. Areas of colocalization giving rise to yellow signal.

Figure 3 - CIT₃ cells expressing EBFP fusion to GLUT1: High power (40x) image of CIT₃ cells expressing EBFP fusion to the C-terminus of GLUT1 (GLUT1-EBFP). The blue fluorescent signal (B) was superimposed on the phase contrast (A) image of the cells to demonstrate the intracellular distribution of GLUT1- EBFP chimera (C), which is otherwise hard to estimate because of the weak signal.

Figure 4: CIT₃ cells expressing EGFP and DsRed1 fusions to the C-terminus of GLUT1: Images captured 48-60 hours after co-transfection with pGLUT1-EGFP and pGLUT1-DsRed1, at x magnification. A, B, C – Cells maintained in GM. D, E, F – Cells exposed to SM for 4 days before capturing the fluorescent signals. A, D – Green fluorescent signals of GLUT1-EGFP at sec. B, E – Red fluorescent signals of GLUT1-DsRed1 at sec. C, F – Combined images. Areas of colocalization giving rise to yellow signal.

Figure 5: Colocalization of GLUT1 green and red fluorescent protein chimeras with native GLUT1. CIT₃ cells transfected with GLUT1-EGFP or GLUT1-DsRed1 were fixed and treated with specific anti-GLUT1 primary antibody, 96 hours after exposure to SM, and 48 hours after

transfection. Texas Red conjugated red antibody and FITC-conjugated green antibody were used as secondary antibodies, respectively. A - The green fluorescent signal of GLUT1-EGFP, B - The red signal of native GLUT1, C - The green fluorescent signal of GLUT1-EGFP co-localized with the red signal of native GLUT1, giving rise to yellow signal. A,B,C - x images with exposure time sec., D - The red fluorescent signal of GLUT1-DsRed1, E - The green signal of native GLUT1, F - The red fluorescent signal of GLUT1-DsRed1 did not co-localize with the green signal of native GLUT1. D,E,F- x images with exposure time sec.

REFERENCES

1. Burnol, A.F., A. Leturque, M. Loizeau, C. Postic and J. Girard. 1990. Glucose transporter expression in rat mammary gland. *Biochem J* 270:277-279.
2. Camps, M., S. Vilaro, X. Testar, M. Palacin and A. Zorzano. 1994. High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. *Endocrinology* 134:924-934.
3. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.
4. Cole, N.B., C.L. Smith, N. Sciaky, M. Terasaki, M. Edidin and J. Lippincott-Schwartz. 1996. Diffusional mobility of Golgi proteins in membranes of living cells. *Science* 273:797-801.
5. Cormack, B.P., R.H. Valdivia and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33-38.
6. Dils, R.R. 1989. Synthetic and secretory processes of lactation. *Proc Nutr Soc* 48:9-15.
7. Dobson, S.P., C. Livingstone, G.W. Gould and J.M. Tavare. 1996. Dynamics of insulin-stimulated translocation of GLUT4 in single living cells visualised using green fluorescent protein. *FEBS Lett* 393:179-184.
8. Drmota, T., G.W. Gould and G. Milligan. 1998. Real time visualization of agonist-mediated redistribution and internalization of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor. *J Biol Chem* 273:24000-24008.
9. Garcia-Mata, R., Z. Bebok, E.J. Sorscher and E.S. Sztul. 1999. Characterization and dynamics of aggresome formation by a cytosolic GFP- chimera. *J Cell Biol* 146:1239-1254.

10. Gould, G.W., V. Derechin, D.E. James, K. Tordjman, S. Ahern, E.M. Gibbs, G.E. Lienhard and M. Mueckler. 1989. Insulin-stimulated translocation of the HepG2/erythrocyte-type glucose transporter expressed in 3T3-L1 adipocytes. *J Biol Chem* 264:2180-2184.
11. Haas, J., E.C. Park and B. Seed. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol* 6:315-324.
12. Heim, R., D.C. Prasher and R.Y. Tsien. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci U S A* 91:12501-12504.
13. Inouye, S. and F.I. Tsuji. 1994. Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett* 341:277-280.
14. Kaether, C., T. Salm, M. Glombik, W. Almers and H.H. Gerdes. 1997. Targeting of green fluorescent protein to neuroendocrine secretory granules: a new tool for real time studies of regulated protein secretion. *Eur J Cell Biol* 74:133-142.
15. Kain, S.R., M. Adams, A. Kondepudi, T.T. Yang, W.W. Ward and P. Kitts. 1995. Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques* 19:650-655.
16. Kida, E., W. Kaczmarski, A.A. Golabek, A. Kaczmarski, M. Michalewski and K.E. Wisniewski. 1999. Analysis of intracellular distribution and trafficking of the CLN3 protein in fusion with the green fluorescent protein in vitro. *Mol Genet Metab* 66:265-271.
17. Lippincott-Schwartz, J., N. Cole and J. Presley. 1998. Unravelling Golgi membrane traffic with green fluorescent protein chimeras. *Trends Cell Biol* 8:16-20.
18. Madon, R.J., S. Martin, A. Davies, H.A. Fawcett, D.J. Flint and S.A. Baldwin. 1990. Identification and characterization of glucose transport proteins in plasma membrane- and Golgi vesicle-enriched fractions prepared from lactating rat mammary gland. *Biochem J* 272:99-105.

19. Matz, M.V., A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Markelov and S.A. Lukyanov. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species [see comments]. *Nat Biotechnol* 17:969-973.

20. Mueckler, M., C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard and H.F. Lodish. 1985. Sequence and structure of a human glucose transporter. *Science* 229:941-945.

21. Nemeth, B.A., S.W. Tsang, R.S. Geske and P.M. Haney. 2000. Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland. *Pediatr Res* 47:444-450.

22. Patterson, G.H., S.M. Knobel, W.D. Sharif, S.R. Kain and D.W. Piston. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J* 73:2782-2790.

23. Presley, J.F., N.B. Cole, T.A. Schroer, K. Hirschberg, K.J. Zaal and J. Lippincott-Schwartz. 1997. ER-to-Golgi transport visualized in living cells [see comments]. *Nature* 389:81-85.

24. Shennan, D.B. 1998. Mammary gland membrane transport systems. *J. Mammary Biol. and Neoplasia*. 3:247-258.

25. Strous, G.J. 1986. Golgi and secreted galactosyltransferase. *CRC Crit Rev Biochem* 21:119-151.

26. Takata, K., K. Fujikura, M. Suzuki, T. Suzuki and H. Hirano. 1997. GLUT1 glucose transporter in the lactating mammary gland in the rat. *Acta Histochem Cytochem* 30:623-628.

27. Toddywalla, V.S., F.W. Kari and M.C. Neville. 1997. Active transport of nitrofurantoin across a mouse mammary epithelial monolayer. *J Pharmacol Exp Ther* 280:669-676.

28. Tsien, R.Y. 1998. The green fluorescent protein. *Annu Rev Biochem* 67:509-544.

29. Wilde, C.J. and N.J. Kuhn. 1981. Lactose synthesis and the utilisation of glucose by rat mammary acini. *Int J Biochem* 13:311-316.

30. Yang, T.T., P. Sinai, G. Green, P.A. Kitts, Y.T. Chen, L. Lybarger, R. Chervenak, G.H. Patterson, D.W. Piston and S.R. Kain. 1998. Improved fluorescence and dual color detection with enhanced blue and green variants of the green fluorescent protein. *J Biol Chem* 273:8212-8216.

31. Zhao, F.Q., W.T. Dixon and J.J. Kennelly. 1996. Localization and gene expression of glucose transporters in bovine mammary gland. *Comp Biochem Physiol B Biochem Mol Biol* 115:127-134.

32. Zhao, F.Q., W.M. Moseley, H.A. Tucker and J.J. Kennelly. 1996. Regulation of glucose transporter gene expression in mammary gland, muscle, and fat of lactating cows by administration of bovine growth hormone and bovine growth hormone-releasing factor. *J Anim Sci* 74:183-189.

Figure 1

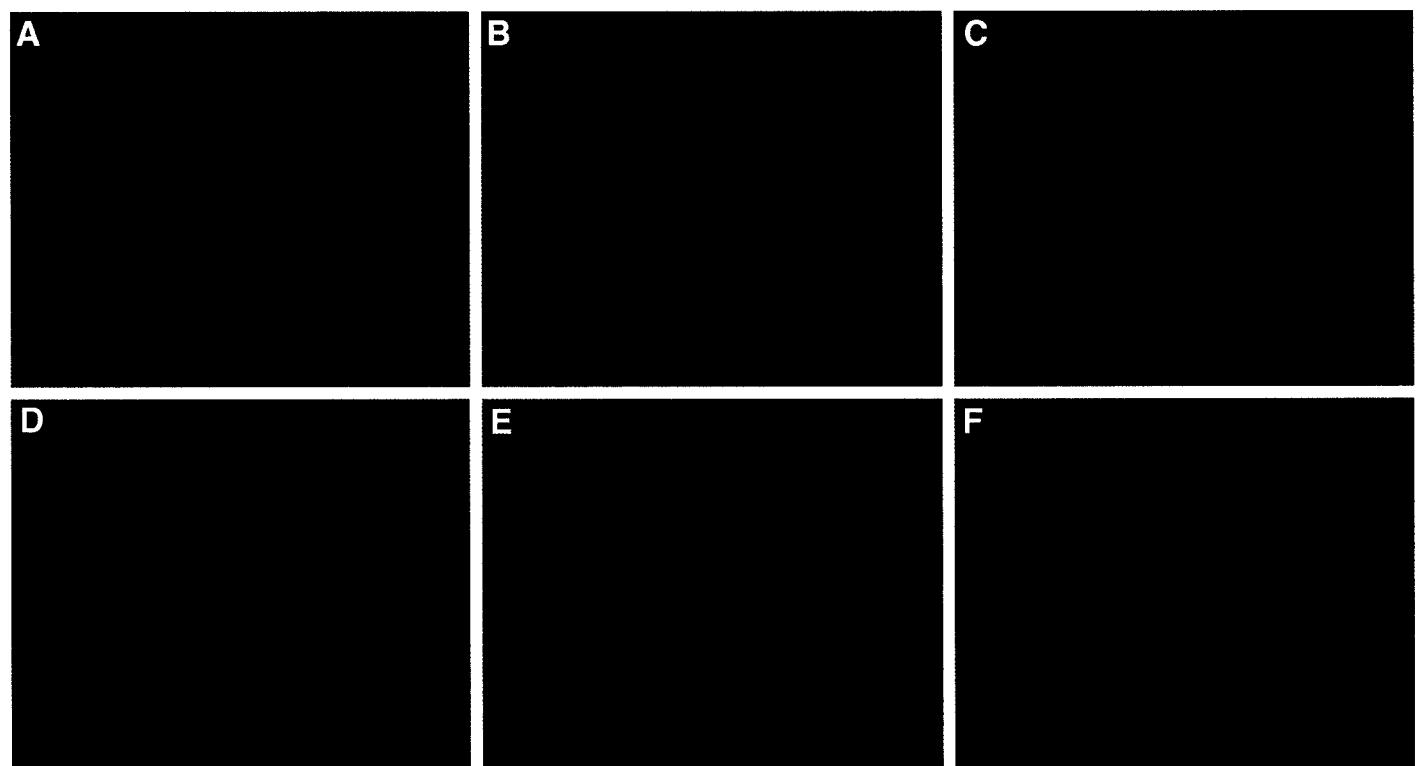


Figure 2



Figure 3



Figure 4

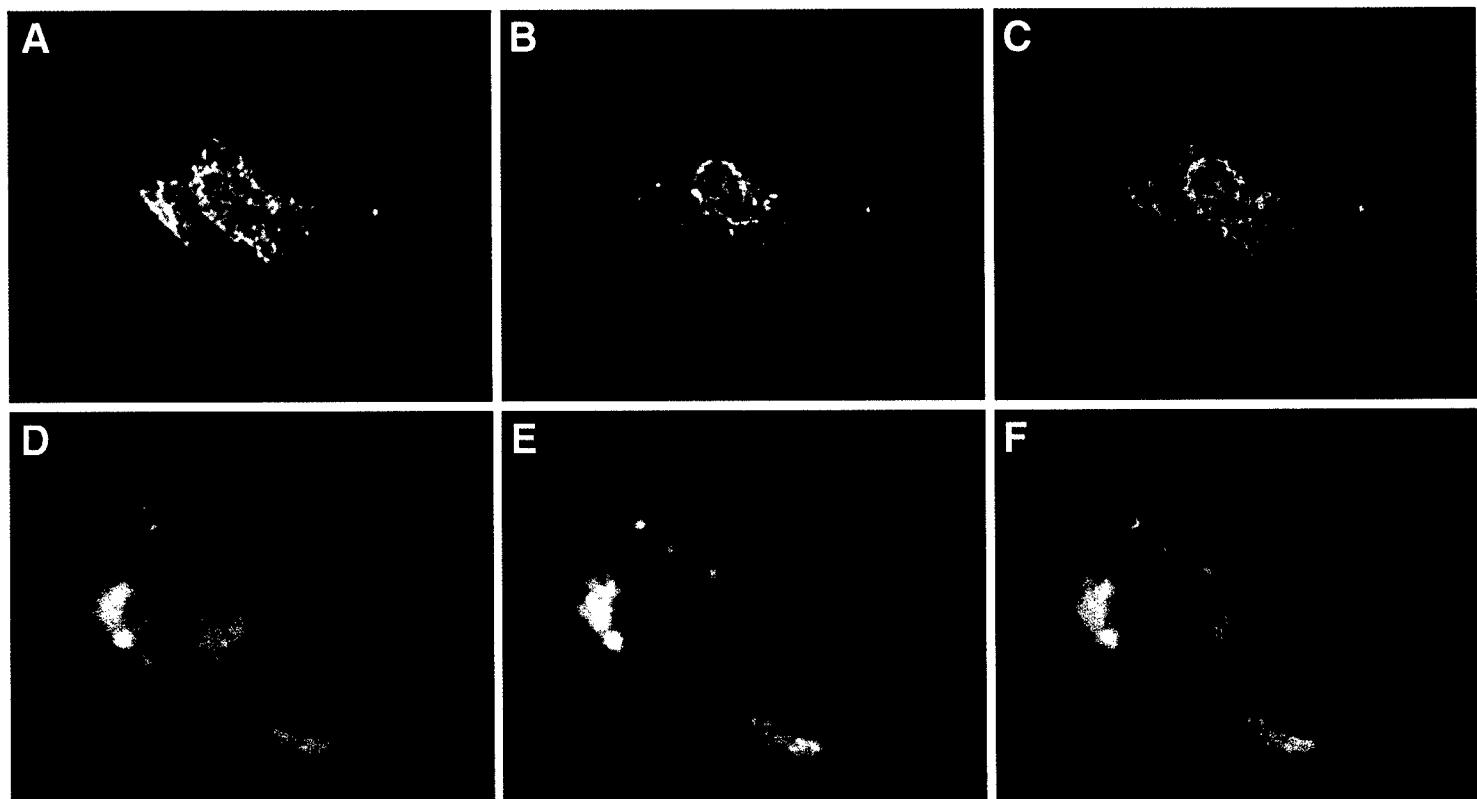
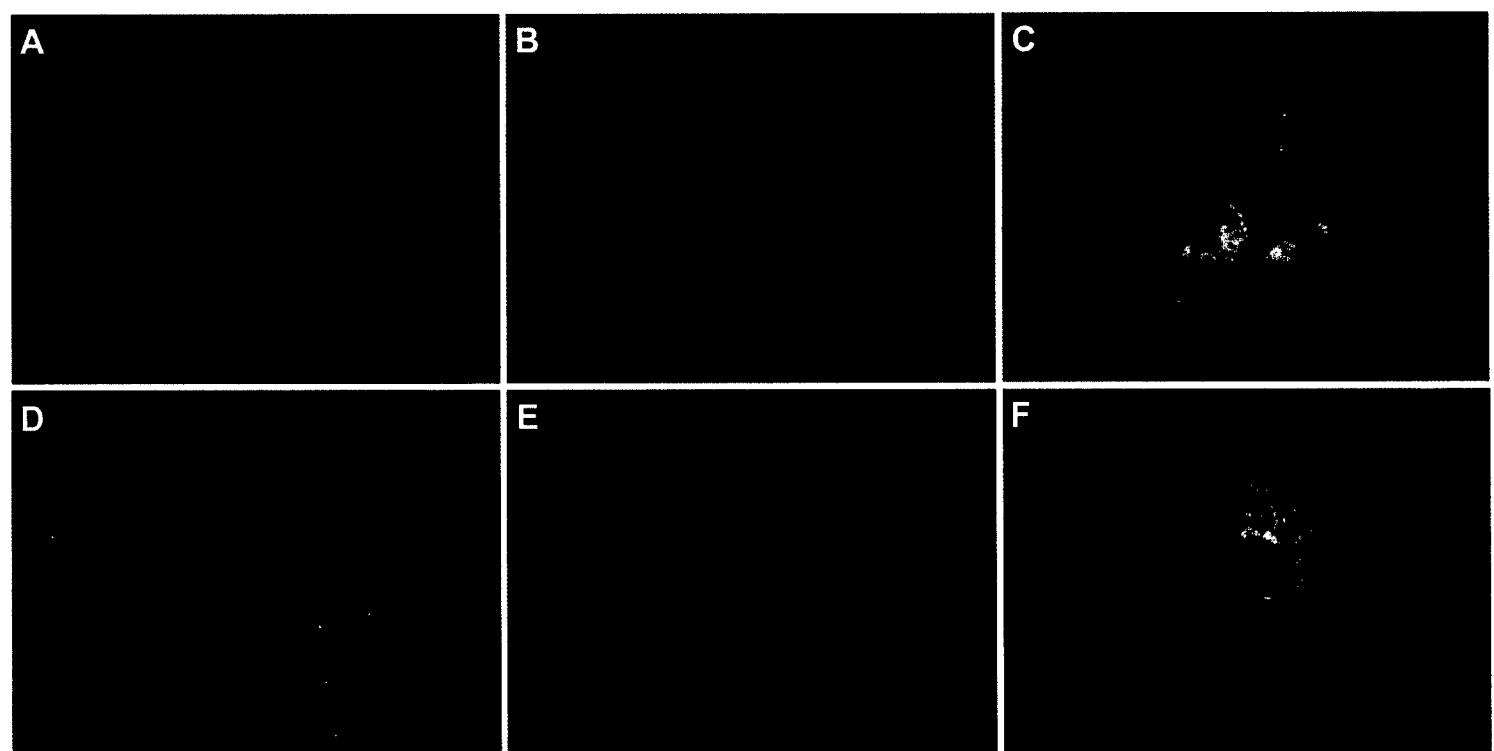


Figure 5



CATIONIC LIPOSOME-MEDIATED STABLE TRANSFECTION OF CIT₃ MOUSE MAMMARY EPITHELIAL CELLS

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Key words: Cationic liposomes / Green Fluorescent Protein (GFP) / Mammary epithelial cells /
Stable transfection

ABSTRACT

Transfection of mammary epithelial cells can provide important insights into the molecular biology of milk secretion and breast cancer. Difficulties transfecting these cells have been reported previously. We compared the relative transfection efficiency of CaPO_4 , DEAE-Dextran, cationic polymers and cationic liposomes. CIT₃ mouse mammary epithelial cells were transfected with green or blue fluorescent protein expression vectors. Transfection efficiency was highest for cationic liposomes compared to all other transfection methods ($p<0.05$). Fluorescence was detected in $31\pm4\%$ of these stably transfected colonies. Cationic liposome-mediated stable transfection efficiently yields stably transfected mammary epithelial cells with a high frequency of exogenous gene expression.

INTRODUCTION

The mammary epithelial cell is a useful model of secretory processes (4, 16), and abnormalities in mammary cell differentiation may be related to the development of breast cancer (12, 14, 15). The expression of exogenous proteins in mammary epithelial cells is an important method in studying such fundamental questions of mammary gland biology.

Transfection introduces foreign DNA into eukaryotic cells in order to study regulation of gene expression, protein targeting, protein processing, protein function, structure-function relationships and pathophysiology. Efficiency of transient and stable transfection varies widely as a function of different cell types, cell lines and promoters (8, 9, 13). The majority of previous studies in mammary epithelial cells, as well as in other cell types, compared transient transfection efficiency, using measures of gene expression, such as β -galactosidase (3, 5, 17), luciferase (2, 3, 17), chloramphenicol acetyltransferase activity (19), fluorescent in situ hybridization (5) or GFP transient expression (2). Recently, Bischof et al. (2) reported successful transient transfection of mouse mammary epithelial cells, but acknowledged long-recognized difficulties encountered in the transfection of this cell type. In their report, which evaluated a PEI/DNA adenovirus system, the usefulness of cationic liposomes was demonstrated by using GFP fluorescence as measure of gene expression in transient transfections.

For many applications, including our intended studies of protein targeting in mouse mammary epithelial cells, stably transfected cells are advantageous. By using stably transfected cells, a population with a homogenous, appropriate, steady-state level of exogenous gene expression can be selected for study (7). Such assessment of stable transfection efficiency in mammary epithelial cells was described previously by Basolo et al. (1), who demonstrated the highest stable transfection efficiency with CaPO_4 , especially when combined with glycerol

shock. However, the results of Basolo et al. were obtained using an immortalized cell line, MCF-10, and did not include a comparison to cationic liposomes or cationic polymers transfection reagents, which became more widely available since that report. Our objective was to determine the most efficient chemical method for stable transfection of mouse mammary epithelial cells with the transfection reagents available today.

MATERIALS AND METHODS

Cell culture: CIT₃ cells are a non-neoplastic cell line derived from mouse mammary epithelial cells. They were selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions and exhibit polarized transport (18). CIT₃ cells were provided by M.C. Neville, Ph.D., University of Colorado School of Medicine. Cells were maintained in growth medium (GM), which is a nutrient-defined basal medium (DMEM/F12, GibcoBRL, Life Technologies Inc., Rockville, MD), containing 10 µg/mL insulin (Sigma, St. Louis, MO) and 5 ng/mL EGF (Epithelial Growth Factor, GibcoBRL, Life Technologies Inc., Rockville, MD).

Comparison of different transfection methods: pEGFP-1 and pEBFP-C1 (Enhanced Green and Blue Fluorescent Protein plasmid vectors, Clontech Laboratories Inc., Palo Alto, CA, #6086-1 and #6070-1, respectively) have a neomycin resistance (Neo^r) gene for geneticin selection in eukaryotic cells. pEBFP has the human CMV immediate early promoter for high-level expression and SV40 origin of replication. A 2.6-kb BamH1 fragment of glucose transporter cDNA (10) was subcloned into the MCS (Multiple Cloning Site) of pEBFP-C1 to create a vector expressing EBFP- GLUT1 fusion protein. In a second construct, pCasproEGFP, provided by D. Hadsell, Ph.D. (Baylor College of Medicine, Houston, TX), a 267-bp Asp718I / BamH1 fragment of mouse β-casein promoter, was subcloned into the MCS of pEGFP-1. Cells transfected with this construct were also treated with prolactin, 3 µg/mL.

The transfection methods employed were:

1. Cationic liposomes (LipoFectAmineTM Reagent, which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and the neutral lipid dioleoyl

phosphatidylethanolamine (DOPE), GibcoBRL, Life Technologies Inc., Rockville, MD, #18324-012),

2. Cationic polymers (SuperFect™ Reagent, which consists of activated-dendrimer molecules that have a defined spherical architecture, with branches that radiate from a central core and terminate at charged amino groups, Qiagen Inc., Valencia, CA, #301305),
3. CaPO₄ (Mammalian Transfection Kit, Stratagene, La Jolla, CA, #200285), and
4. DEAE-Dextran (Mammalian Transfection Kit, Stratagene, La Jolla, CA, #200285).

Transfection of 2 μ g DNA was performed in 35-mm dishes containing 5×10^5 cells per plate. All transfection reagents were used according to the manufacturer's instructions. After 48h, cells were split 1:8. Selective medium containing 0.5 mg/mL geneticin (GibcoBRL, Life Technologies Inc., Rockville, MD) was added 24h later. 0.5 mg/mL geneticin was determined as the optimal concentration required to kill 80-90% of control cells within 4d (data not shown). Cells were maintained in selective media containing geneticin until the colonies were counted 17d after transfection. This time interval was chosen, based on previous experiments (data not shown), showing new colonies forming and starting to express fluorescence up to 14d after adding geneticin to the medium. Stable transfection efficiency was determined by the number of surviving colonies with diameter of 1-mm or greater per 100-mm plate. Dishes were photographed using a Cohu 4910 uncooled CCD camera and quantitated using image processing software (Image Tool, UTHSCSA, San Antonio, TX). Statistical analysis was by one way ANOVA (SigmaStat, version 2.03).

Measurement of the frequency of exogenous gene expression in stable colonies transfected with cationic liposomes:

For this purpose, cells were transfected with pEGFP-N1 (Clontech Laboratories Inc., Palo Alto, CA, # 6085-1). This vector has the Neo^r gene for geneticin selection and the human CMV immediate early promoter.

Transfection of 2 μ g pEGFP-N1 DNA, diluted in 100 μ L basal serum-free medium, DMEM/F12, was performed in 35-mm dishes containing 5×10^5 cells per plate (60-80% confluent). Cationic liposomes (LipoFectAmineTM, GibcoBRL, Life Technologies Inc., Rockville, MD, #18324-012) transfection reagent (20 μ L diluted in 100 μ L DMEM/F12 per reaction) was combined with the diluted DNA and incubated for 30min in room temperature to allow DNA-liposome complexes to form. While complexes were forming, the cells were rinsed once with DMEM/F12. A total of 200 μ L of the DNA-liposome complex solution was mixed with 800 μ L of GM without antibiotics and added to the cells. Cells were incubated in this transfection medium for 5h in 37°C in a CO₂ incubator, before 1 mL of full GM was added to each plate. Medium was replaced after 24h. After 48h, cells were split 1:8. Selective medium containing 0.5mg/mL geneticin was added 24h later. Cells were maintained in selective media containing geneticin until the colonies were checked 17d after transfection. EGFP has an excitation maximum of 488 nm and an emission maximum of 507 nm. Fluorescent signal was detected using an OLYMPUS iX-70 microscope. Data were captured by an uncooled color CCD camera (Optronics, DEI-750 CE Digital Output Model S60675). The total number of colonies \geq 1-mm in diameter and the number of these colonies expressing GFP were counted.

RESULTS

Comparison of different transfection methods:

Transfection efficiency was expressed as number of colonies \geq 1-mm in diameter per 100-mm plate. Efficiency did not differ significantly between the two vectors we used for these experiments. This threshold was selected because colonies of this size can consistently be transferred and expanded for further study. Liposome-mediated transfection was 40-60% more efficient in mouse mammary epithelial cells compared to all other transfection methods ($p<0.05$) (Figure 1). Cationic polymers were also consistently useful, although less efficient. CaPO₄ was not consistent. DEAE-Dextran was not suitable for stable transfection of mammary epithelial cells, yielding no colonies at all.

The mean area of the individual colonies was similar and independent of the transfection method employed, implying that cell growth is independent of transfection method (Figure 2). This finding permits the use of number of colonies as a comparison measure for efficiency in stable transfections done by different methods.

Direct initial toxicity from the transfection procedure was highest for cationic liposomes and cationic polymers and lowest for CaPO₄ and DEAE-Dextran. This was estimated based on the percentage of cell detachment within 24h after transfection (approximately 40-50% for cationic liposomes, 35-40% for cationic polymers, 10-20% for CaPO₄ and only 0-10% for DEAE-Dextran).

Cost per transfection (based on the published prices of the different kits used), and convenience of use (based on our assessment using the different kits, according to the manufacturers' recommendations) were similar for all methods.

Measurement of the frequency of exogenous gene expression in stable colonies transfected with cationic liposomes:

We used EGFP, which is more easily detected than EBFP (11), to measure the frequency of exogenous gene expression in stable colonies transfected with cationic liposomes. For this purpose, cells were transfected with pEGFP-N1. This vector has the Neo^r gene for geneticin selection and the human CMV immediate early promoter, which we expected to provide a higher level of expression than we had observed with the β -casein promoter.

In three independent stable transfections, $31 \pm 4\%$ (mean \pm S.E.M.) of the colonies emitted green fluorescent signal (Table 1). Expression levels within a given colony were similar, and only infrequent cells had high or absent expression (Fig. 3 B). GFP had the expected subcellular localization (6) throughout the cell, with exclusion only of the nucleoli (Fig. 3 D).

DISCUSSION

In this study we judged transfection efficiency by measure of stably transfected colonies, which incorporated the Neo^r (geneticin resistance) gene, and thus became resistant to the selective medium. The majority of previous studies in mammary epithelial cells, as well as in other cell types, compared transient transfection efficiency, using measures of gene expression. The number of stable colonies in selective medium is a direct measure of transfection efficiency, and is unrelated to factors that may influence expression of the exogenous gene of interest. Stable transfection also provides an important tool for experiments aimed at studying a population of cells with a uniform and appropriate level of exogenous gene expression. Only Basolo et al. (1) previously described this approach in mammary epithelial cells. Our results for stable transfections, demonstrating highest transfection efficiency in mammary epithelial cells using cationic liposomes, supersede those of Basolo et al. (1). On the other hand, our results complement those of Bischof et al. (2), who also demonstrated the usefulness of cationic liposomes, but did so in transient transfections.

The large number of stable colonies transfected with cationic liposomes together with the high frequency of exogenous gene expression, as measured by EGFP expression, enable easy passage of any number of selected stable colonies expressing the gene of interest for further study. In summary, cationic liposomes provide a consistently efficient, cost-effective, and convenient method for stable transfection of mammary epithelial cells. The ease and reliability with which stable colonies expressing an exogenous gene can be prepared should result in wider use of this method by mammary gland biologists, yielding important insights into milk secretion and breast cancer.

REFERENCES

1. Basolo, F., J. Elliott and J. Russo. 1990. Transfection of human breast epithelial cells with foreign DNA using different transfecting techniques. *Tumori* 76:455-460.
2. Bischof, J., I. Vietor, M. Cotten and L.A. Huber. 1999. Transient transfection of mammary epithelial cells with a PEI/DNA/adenovirus system. *Biol. Chem.* 380:269-273.
3. Budker, V., J.E. Hagstrom, O. Lapina, D. Eifrig, J. Fritz and J.A. Wolff. 1997. Protein/amphipathic polyamine complexes enable highly efficient transfection with minimal toxicity. *BioTechniques* 23:139, 142-137.
4. Burgoyne, R.D. and C.J. Wilde. 1994. Control of secretory function in mammary epithelial cells. *Cell Signal* 6:607-616.
5. Cheng, L., C.D. Bucana and Q. Wei. 1996. Fluorescence *in situ* hybridization method for measuring transfection efficiency. *BioTechniques* 21:486-491.
6. Dobson, S.P., C. Livingstone, G.W. Gould and J.M. Tavare. 1996. Dynamics of insulin-stimulated translocation of GLUT4 in single living cells visualised using green fluorescent protein. *FEBS Lett.* 393:179-184.
7. Haney, P.M., M.A. Levy, M.S. Strube and M. Mueckler. 1995. Insulin-sensitive targeting of the GLUT4 glucose transporter in L6 myoblasts is conferred by its COOH-terminal cytoplasmic tail. *J. Cell Biol.* 129:641-658.
8. Kane, S.E. 1991. High-level expression of foreign genes in mammalian cells. *Genet. Eng.* 13:167-182.
9. Kucherlapati, R. and A.I. Skoultchi. 1984. Introduction of purified genes into mammalian cells. *CRC Crit. Rev. Biochem.* 16:349-379.

10. Mueckler, M., C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard and H.F. Lodish. 1985. Sequence and structure of a human glucose transporter. *Science* 229:941-945.
11. Patterson, G.H., S.M. Knobel, W.D. Sharif, S.R. Kain and D.W. Piston. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782-2790.
12. Petersen, O.W., L. Ronnov-Jessen, V.M. Weaver and M.J. Bissell. 1998. Differentiation and cancer in the mammary gland: shedding light on an old dichotomy. *Adv. Cancer Res.* 75:135-161.
13. Ray, J. and F.H. Gage. 1992. Gene transfer into established and primary fibroblast cell lines: comparison of transfection methods and promoters. *BioTechniques* 13:598-603.
14. Rudland, P.S. and R. Barraclough. 1988. Stem cells in mammary gland differentiation and cancer. *J. Cell Sci. Suppl.* 10:95-114.
15. Russo, J. and I.H. Russo. 1997. Differentiation and breast cancer. *Medicina (B Aires)* 57:81-91.
16. Shennan, D.B. 1998. Mammary gland membrane transport systems. *J. Mammary Biol. Neoplasia*. 3:247-258.
17. Thompson, C.D., M.R. Frazier-Jessen, R. Rawat, R.P. Nordan and R.T. Brown. 1999. Evaluation of methods for transient transfection of a murine macrophage cell line, RAW 264.7. *BioTechniques* 27:824-826, 828-830, 832.
18. Toddywalla, V.S., F.W. Kari and M.C. Neville. 1997. Active transport of nitrofurantoin across a mouse mammary epithelial monolayer. *J. Pharmacol. Exp. Ther.* 280:669-676.

19. Verma, R.S., D. Giannola, W. Shlomchik and S.G. Emerson. 1998. Increased efficiency of liposome-mediated transfection by volume reduction and centrifugation. *BioTechniques* 25:46-49.

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Experiment	Number of colonies \geq 1mm per 100-mm dish	Number of colonies expressing GFP	Percent colonies expressing GFP (%)
1	176	42	24
2	132	41	31
3	147	54	37
Mean \pm S.E.M.	152 \pm 13	46 \pm 4	31 \pm 4

Table 1. Level of gene expression in stable colonies transfected by cationic liposomes, based on the percent of colonies expressing GFP. Transfection of pEGFP-N1 DNA using cationic liposomes (LipoFectAmineTM) transfection reagent was used as described in text. The total number of colonies \geq 1mm in diameter per 100-mm plate, and the number of these colonies expressing GFP, were counted 17d after transfection.

Figure 1. Transfection efficiency expressed as number of colonies \geq 1-mm in diameter per 100-mm plate as counted 17 days after transfection. Liposome-mediated transfection was consistently the most efficient compared to other methods ($p < 0.05$, one way ANOVA). DEAE-Dextran yielded no colonies in any experiment. In experiment 1, transfection of pEBFP-GLUT1 with CaPO₄ yielded no colonies.

Figure 2. The area of the individual colonies was similar and independent of the transfection method employed: The mean area of the colonies counted on day 17 post-transfection (Fig. 1) is shown. Error bars indicate standard errors of the mean. Statistical analysis was by one way ANOVA. There was no statistically significant difference between the areas of the colonies under different transfection methods. In experiment 1, transfection of pEBFP-GLUT1 with CaPO₄ yielded no colonies.

Figure 3. Stable colonies transfected by cationic liposomes expressing GFP. Transfection of 2 μ g pEGFP-N1 DNA was performed in 35-mm dishes containing 5×10^5 cells per plate. Cationic liposomes (LipoFectAmine) transfection reagent was used as described in text. Fluorescent signal data were captured by an uncooled color CCD camera, and enhanced in a linear manner using Adobe Photoshop 5.0. Green fluorescent signal is demonstrated on black-white scale, white being the strongest green signal. A – Low power magnification (100X)(Bar, 60 μ m). B – High power magnification (600X)(Bar, 10 μ m). A, C – Phase contrast. B, D – Green fluorescent signal. The low power magnification (B) shows most cells within a narrow range of green fluorescent signal; few cells show high or absent expression. The high power magnification (D) shows, as expected (6), that GFP is distributed throughout the cell with exclusion only of the nucleoli.

FIGURE 1

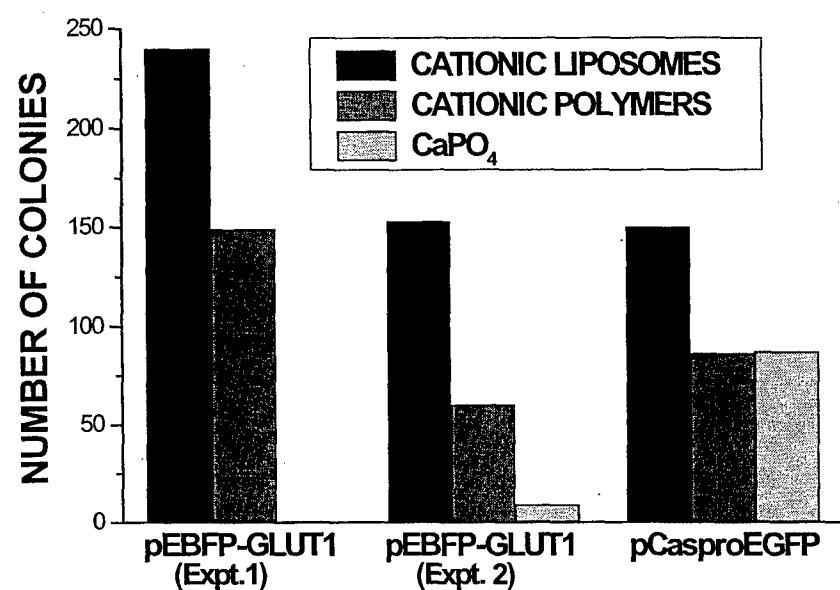


FIGURE 2

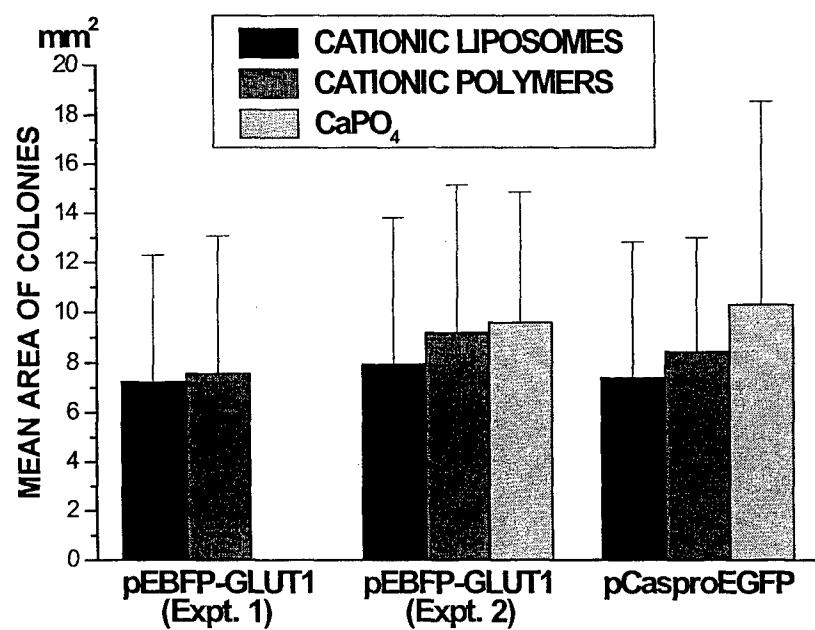
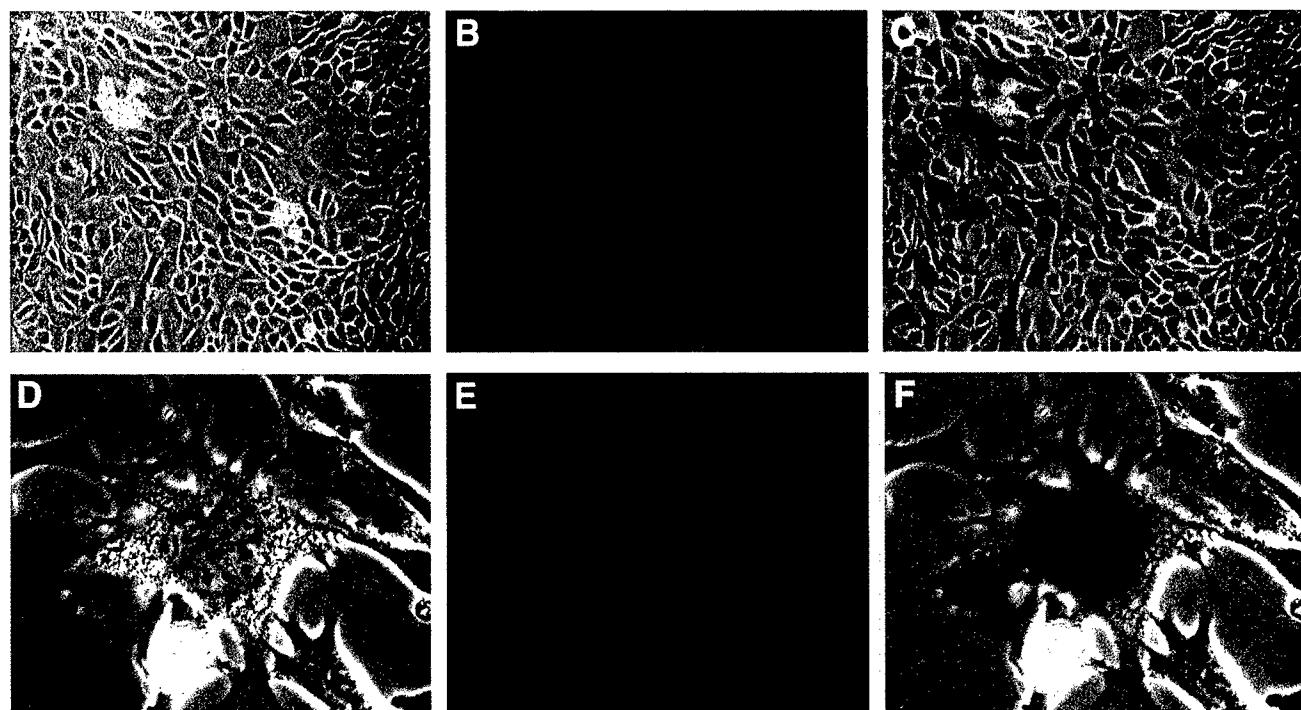


FIGURE 3



**DYNAMIC HORMONALLY-REGULATED TARGETING OF THE GLUT1
GLUCOSE TRANSPORTER IN MOUSE MAMMARY EPITHELIAL CELLS**

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Key words: mouse mammary epithelial cells, green fluorescent protein (GFP), GLUT1,
prolactin, bafilomycin A1, wortmannin, staurosporine.

ABSTRACT

The mammary gland is unique in its requirement for transport of free glucose into the Golgi, the site of lactose synthesis. GLUT1 is the only known isoform of glucose transporter expressed in the mammary gland. Lactogenic hormones cause targeting of GLUT1 to the Golgi. Mechanisms of intracellular targeting of GLUT1 are not known.

Hypothesis: GLUT1 intracellular targeting under hormonal stimulation is dynamic.

Methods: To construct fusion proteins of GLUT1 and green fluorescent protein (GFP), cDNA was subcloned into pEGFP-C1 and pEGFP-N1 (Clontech). GFP-GLUT1 fusion proteins were expressed in CIT₃ mouse mammary epithelial cells (M.C. Neville, University of Colorado) using liposome-mediated transfection. Cells were maintained in growth medium, or exposed to prolactin at different concentrations and for different times. To further define the intracellular trafficking mechanisms involved in GLUT1 targeting and recycling in mammary epithelial cells, cells were also exposed to inhibitors, which affect GLUT1 and GLUT4 targeting in muscle and fat cells, including bafilomycin A1, wortmannin and staurosporine. Cells were studied at 37°C. Time lapse fluorescent images were captured by an uncooled CCD camera.

Results: In growth medium, the N- and the C-terminal fusions of GFP and GLUT1 demonstrated plasma membrane targeting. Prolactin changed subcellular targeting of both fusion proteins to an intracellular punctate pattern, as seen with native GLUT1. Time lapse images revealed dynamic trafficking of GFP-GLUT1 fusion proteins. Upon exposure to prolactin, GLUT1 fusion proteins were redistributed intracellularly, starting after approximately 50-60 minutes, with maximal intracellular targeting within 90-110 minutes. When the cells were returned to GM, most of the changes were reversible.

Bafilomycin A1, which causes arrest of endosomal acidification, caused central coalescence of GFP-GLUT1 and the loss of peripheral vesicles. Wortmannin and staurosporine effects on internalization of GLUT1 were not specific to mammary epithelial cells or prolactin exposure, but supported basal recycling of GLUT1.

Conclusions: Our results demonstrate a basal constitutive GLUT1 membrane-recycling pathway between an intracellular pool and the cell surface in mouse mammary epithelial cells, which targets most of the GLUT1 to the plasma membrane in maintenance medium. Upon exposure to prolactin GLUT1 is specifically targeted intracellularly. This change takes place within hours, and may support glucose transport as a rate-limiting step for lactose synthesis during lactation. Arrest of endosomal acidification by bafilomycin A1 disrupts this process, which implies trafficking via endosomal pathways.

INTRODUCTION

Lactose is the major osmotic constituent of human milk and thus the major determinant of the volume of milk produced. The mammary gland is unique in its requirement for transport of free glucose into the Golgi, which is the site of lactose synthesis (Strous, 1986). GLUT1 is the only known isoform of glucose transporters expressed in the mammary gland (Burnol et al., 1990; Camps et al., 1994; Shennan, 1998). In most cells GLUT1 is localized to the plasma membrane and is responsible for basal glucose uptake. Evidence from *in vivo* and *in vitro* studies demonstrates unique hormonally regulated subcellular targeting of GLUT1 to Golgi as well as plasma membrane in mouse mammary epithelial cells (Madon et al., 1990; Nemeth et al., 2000; Takata et al., 1997).

The aim of this work was to study the effects of lactogenic hormones, namely prolactin, on the subcellular targeting of GLUT1 from the basolateral membrane intracellularly. Our hypothesis, based on our prior work (Nemeth et al., 2000), was that GLUT1 targeting under hormonal stimulation is a dynamic process, which takes place within minutes to hours and is reversible. In an effort to further define the intracellular trafficking mechanisms involved in GLUT1 targeting and recycling in mammary epithelial cells, agents which affect GLUT1 and GLUT4 targeting in muscle and fat cells were used.

MATERIALS AND METHODS

Subcloning GLUT1 cDNA into Green Fluorescent Protein (GFP) plasmid vectors:

pEGFP-C1 and pEGFP-N1 green fluorescent protein plasmid vectors (#6084-1 and #6085-1, respectively, Clontech Laboratories Inc., Palo Alto, CA) were used.

GLUT1 cDNA (Mueckler et al., 1985) was recovered from pHepG2 using Bam H1 restriction digest or PCR. The primers for the PCR reaction were designed to include Hind III and Bam H1 restriction sites at the N- and C-terminus of GLUT1 cDNA, respectively. Restriction digest of the insert and the plasmid vector multiple cloning site with these enzymes followed by ligation allowed GLUT1 cDNA to be subcloned in the right orientation. GLUT1 cDNA was subcloned into pEGFP-C1 and pEGFP-N1 respectively, to create N- and C-terminus fusion of GLUT1 to green fluorescent protein. The N-terminus fusion of GLUT1 to EGFP was also constructed without PCR, subcloning GLUT1 cDNA, recovered by Bam H1 restriction digest, into Bam H1 site of pEGFP-C1. This could not be done for the C-terminus fusion of GLUT1 cDNA due to an intervening stop codon. All the recombinant vectors were sequenced to verify the right orientation and exclude mutations.

Cell culture and medium:

CIT₃ cells were provided by M.C. Neville, PhD., University of Colorado School of Medicine. Cells were maintained in growth medium (GM), which is a nutrient-defined basal medium (DMEM/F12), containing 10 μ g/ml insulin and 5ng/ml EGF. To stimulate differentiation by lactogenic hormones, the media was changed to secretion medium (SM), by adding prolactin 3 μ g/ml and hydrocortisone 3 μ g/ml, and withdrawing EGF.

The routine exposure to SM was 96 hours prior to evaluating changes in GLUT1 subcellular targeting.

Transfection:

Transient transfections were used to introduce the recombinant vectors carrying GFP fusion to GLUT1 into the cells. Liposome-mediated transfection using LipoFectAmine Plus Reagent (#10964013, GibcoBRL, Life Technologies Inc., Rockville, MD) was performed in 35-mm dishes, containing 5×10^5 cells per plate (60-80% confluent). 1 μ g DNA was diluted in basal serum-free medium, DMEM/F12, and 6 μ l of premixed Plus reagent was added to give final volume of 100 μ l. The mixture was incubated at room temperature for 15 minutes to allow DNA-Plus complexes to form. Cationic liposomes (LipoFectAmine) transfection reagent (4 μ l diluted in 100 μ l DMEM/F12) was combined with the diluted DNA-Plus mixture, and incubated for 15 minutes in room temperature to allow DNA-Plus-liposome complexes to form. While complexes were forming, the medium was replaced with 800 μ l of GM / SM without antibiotics. 200 μ l of the DNA-Plus-liposome complexes solution was added to this medium and gently mixed into it. Cells were incubated in this transfection medium for 3 hours in 37°C in a CO₂ incubator, before 1 ml of full GM / SM was added to the plate. Transient transfections were checked for fluorescent signal at 48-72 hours, when maximal expression of the fluorescent signal was noted in 20-30% of the cells, based on previous experiments (data not shown).

Immunocytochemistry:

For immunocytochemistry, cells were grown on glass coverslips and fixed. Cells were permeabilized with 0.1% Triton X-100 in PBS. Treatment with primary antibody in

0.1% horse serum in PBS was performed overnight at 4°C. Peptide-affinity purified mouse polyclonal antibody against the C-terminus of GLUT1 (Gould et al., 1989), 6 µg/ml, were used as primary antibodies. FITC-conjugated goat anti-rabbit antibody and Texas Red conjugated rabbit anti-mouse antibody (diluted 1:100 with 0.1% horse serum in PBS) were used as secondary antibodies. Coverslips were then mounted in Pro Long anti-fade medium (Molecular Probes, Eugene, OR) on glass slides for microscopic examination.

Fluorescent microscopy:

Fluorescent signal was detected using OLYMPUS iX-70 microscope. Images were captured by an uncooled color CCD camera (Optronics, DEI-750 CE Digital Output Model S60675). Exposure was adjusted in a linear manner and separate colour channels were merged as indicated using Adobe Photoshop 5.0. For the study of changes taking place in cells over time and under different conditions, cells were grown on round coverslips, and were maintained in a 37°C chamber (). Time-lapse images were captured every 2-5 minutes (depending on the rate of changes) using Snappy software (). After linear adjustments of the images in Photoshop 5.0, as described above, the time-lapse images were combined into a sequence using Flash 4.0 software. Each condition was studied at least 3 times and results were consistent in all cases. Representative results are shown.

Hormones and inhibitors:

Cells transfected with GLUT1-EGFP and kept in GM were exposed to SM. Time lapse images of the changes taking place in GLUT1-EGFP fluorescent signal subcellular targeting were recorded. Cells in GM were afterwards exposed to secretion medium

without hydrocortisone, containing different concentrations of prolactin (3 μ g/ml as in full SM, as well as lower concentrations of 300, 30 and 3 ng/ml). To exclude the possibility of a hydrocortisone effect, cells in GM were exposed to secretion medium containing hydrocortisone (3 μ g/ml, as in full SM), but no prolactin. Cells kept in GM or exposed to SM for 96 hours were exposed to bafilomycin A1 (800nM), wortmannin (1 μ M), or staurosporine (2 μ M). Optimal concentrations of the inhibitors were determined based on the range of concentrations cited in other studies and pre-testing different concentrations within that range. Time lapse images of the changes taking place in GLUT1-EGFP fluorescent signal subcellular targeting were recorded.

RESULTS

GLUT1 fusion chimeras to EGFP exhibit normal GLUT1 targeting in vitro:

GLUT1 cDNA sequence was recovered from pHepG2 by BamH1 restriction digest or PCR, and was subcloned into pEGFP to create N- and C-terminus fusions. The recombinant plasmid vectors were introduced into CIT₃ cells by transient liposome-mediated transfection, achieving fluorescent expression in 20-30% of the cells.

EGFP chimeras to GLUT1 demonstrate change in subcellular distribution from the basolateral plasma membrane, when maintained GM, into the cell, after 96 hours exposure to SM (Fig. 1). Perinuclear distribution with punctate pattern scattered through the cytoplasm is seen in SM. Both the N- and C-terminus fusions to GLUT1 (EGFP-GLUT1 and GLUT1-EGFP, respectively) exhibit the same targeting patterns (Fig. 1).

Immunocytochemistry studies with antibodies against the C-terminus of native GLUT1 showed that the fluorescent signal of both GLUT1 chimeras to GFP co-localized with native GLUT1 (Fig. 2)

GLUT1 intracellular trafficking in SM is dynamic:

Static images of cells at different levels of differentiation in SM demonstrate that dynamic transport systems are involved in the trafficking of GFP-GLUT1 fusion proteins (Fig. 3). This is fully demonstrated when living CIT₃ cells transfected with GLUT1-EGFP and kept in SM are followed by time-lapse imaging, where dynamic trafficking of GLUT1 fusion proteins can be seen (Fig. 4, also available as movie).

Prolactin induces GLUT1 intracellular targeting in living mammary cells:

When living CIT3 cells, transfected with GLUT1-EGFP and kept in GM, are exposed to SM, dynamic trafficking of GLUT1 fusion proteins intracellularly is

demonstrated, starting after approximately 50-60 minutes, with maximal intracellular targeting within 90-110 minutes. When the cells are returned to GM, most of the changes are reversible within 1-2 hours, although not fully, with redistribution of the fluorescent GLUT1 chimera mostly in the plasma membrane (Fig. 5, also available as movie).

Exposure of CIT₃ cells kept in GM to secretion medium containing prolactin without hydrocortisone caused the same changes in GLUT1 subcellular targeting. The same response was reproduced with prolactin concentrations as low as 3 ng/ml (compared to the 3 µg/ml of prolactin usually used in SM) (Fig. 6, also available as a movie). We were not able to demonstrate dose-response relations with the different prolactin concentrations, possibly because the results are qualitative, rather than quantitative. There was no difference in the time required to achieve maximal effect with different prolactin concentrations.

Secretion medium containing hydrocortisone (3 µg/ml as in full SM) without any prolactin caused no change in GLUT1 subcellular distribution (Fig. 7, also available as a movie).

Bafilomycin A1 causes central coalescence of GLUT1-EGFP with loss of peripheral vesicles in SM:

CIT₃ transfected with GLUT1-EGFP and kept in SM for 96 hours show central coalescence of GLUT1-EGFP green signal with the loss of peripheral vesicles after treatment with bafilomycin A1 (Fig. 8-9, also available as movies). The effect was seen after approximately 1 hour and was not reversible upon withdrawal of bafilomycin A1.

Wortmannin causes internalization of GLUT1 signal in GM:

In GM wortmannin causes internalization of GLUT1-EGFP plasma membrane signal (Fig. 10, also available as a movie). The effect was seen after approximately 1-2 hours. No reversibility of the effect could be demonstrated upon withdrawal of the wortmannin.

Staurosporine causes internalization of GLUT1-EGFP in GM and central coalescence of its vesicles in SM:

In GM staurosporine causes internalization of GLUT1-EGFP plasma membrane signal (Fig. 11, also available as a movie). This set of time lapse images also suggests a possible intracellular and intercellular network which constitutes the pathway for internalization of the GLUT1-EGFP vesicles. In cells exposed to SM for 96 hours, staurosporine causes central coalescence of GLUT1-EGFP signal and loss of peripheral vesicles signal (Fig. 12, also available as a movie).

DISCUSSION

A primary function of the differentiated mammary epithelial cell is the synthesis of lactose, within the Golgi, from UDP-galactose and free glucose. The final step in the biosynthesis of lactose is catalyzed by lactose synthetase, a complex of α -lactalbumin and the Golgi enzyme β 1,4-galactosyltransferase(Strous, 1986). The mammary gland is unique in its requirement for transport of free glucose into the Golgi. GLUT1, the only glucose transporter expressed in mammary epithelial cells(Burnol et al., 1990; Camps et al., 1994; Shennan, 1998), normally resides in the plasma membrane. Hormonally regulated subcellular targeting of GLUT1 may have an important role for lactose synthesis in the Golgi of the mammary epithelial cell during lactation. Previously, the role of GLUT1 in glucose transport into Golgi of rodents has been controversial (Camps et al., 1994; Madon et al., 1990; Takata et al., 1997). However, recently published evidence demonstrates unique hormonally regulated subcellular targeting of GLUT1 from the plasma membrane to Golgi in mouse mammary gland during lactation (Nemeth et al., 2000). The aim of this work was to further study the effects of lactogenic hormones on the intracellular trafficking and targeting of GLUT1. The need to study in vitro a dynamic process, which takes place within hours and is reversible, required a system of living cells with labeling of GLUT1.

Green Fluorescent Protein (GFP) is a reporter molecule for monitoring gene expression and protein localization in vivo, in situ and in real time (Chalfie et al., 1994; Dobson et al., 1996; Drmota et al., 1998; Kain et al., 1995; Lippincott-Schwartz et al., 1998; Presley et al., 1997a; Tsien, 1998). GFP is expressed in eukaryotic cells as a fusion protein that serves as a “fluorescent tag”. The use of fluorescent fusion proteins of

GLUT1 allows the study of the same cells over time, permits studies of exocytosis and endocytosis, and not just steady state distributions. It also permits targeting of chimeric proteins to be evaluated in an antibody-independent fashion, and confirms that we are studying GLUT1, and not a novel, lactation-specific Golgi glucose transporter isoform that shares the GLUT1 epitope.

For these properties, an in vitro system using GFP fusion to GLUT1 is ideal for studying the intracellular trafficking and subcellular targeting of GLUT1 in mammary epithelial cells under hormonal stimulation. This system can test the hypothesis that this is a dynamic process, which takes place within hours and is reversible.

EGFP (Enhanced GFP) carries a red-shifted variant of wild-type GFP, which has been optimized for brighter green fluorescence and higher expression in mammalian cells. It has excitation maximum of 488 nm and emission maximum of 507 nm. EGFP contains two amino acid substitutions (Phe-64 to Leu and Ser-65 to Thr) (Cormack et al., 1996; Patterson et al., 1997; Yang et al., 1998). Its usefulness as a fluorescent 'tag' in dynamic intracellular trafficking and targeting studies was reported (Garcia-Mata et al., 1999; Kaether et al., 1997; Kida et al., 1999). This vector has the human CMV immediate early promoter for high level expression. To ensure maximal mammalian expression, the coding regions of pEGFP contain more than 190 silent base mutations, which correspond to human codon-usage preferences (Haas et al., 1996).

CIT₃ cells are a non-neoplastic cell line derived from mouse mammary epithelial cells (after being selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions and exhibit polarized transport) (Toddywalla et al., 1997).

GLUT1 cDNA sequence (Mueckler et al., 1985) was subcloned into pEGFP and introduced into CIT₃ cells by transient transfection, with over-expression of the fluorescent GLUT1 in approximately quarter of the cells. The intracellular targeting of GLUT1-EGFP chimera was consistent from cell to cell.

Prolactin in SM changes subcellular targeting of GFP fusion chimeras to GLUT1 from a plasma membrane distribution to an intracellular perinuclear punctate pattern (Fig. 1). The fact that this pattern was consistent with the distribution of native GLUT1 (Fig.2) support the use of GLUT1 chimeras to GFP as a model for studying GLUT1 intracellular targeting in mammary epithelial cells. Since the behavior and intracellular distribution of both the N- and C-fusion chimeras of GLUT1 to GFP were consistently the same (Fig. 1), further studies were carried out with only one of them (GLUT1-EGFP).

Living mouse mammary epithelial cells in SM demonstrate dynamic trafficking of GLUT1 fusion proteins to GFP, especially when multiple time-lapse images are captured (Fig. 3, compared to Fig. 4, which is available also as a movie). Carefully following these green fluorescent GLUT1 vesicles excludes random movement, and actually suggests that the dynamic intracellular targeting of GLUT1 may be mediated through altering GLUT1 exocytosis and endocytosis.

When CIT₃ cells kept in GM were exposed to SM, the changes in GLUT1 targeting from mostly plasma membrane pattern to intracellular pattern occurred within 60-120 minutes. The maximal intracellular translocation of GLUT1-EGFP green fluorescent signal was noted at 100-110 minutes after exposure to the lactogenic hormones in SM (fig. 5, also available as a movie). Some of this effect was reversible within 60-120 minutes upon withdrawal of SM, and replacement in GM. We were not

able to demonstrate full reversibility of the process in this *in vitro* system. The relatively quick changes in GLUT1 targeting in living mouse mammary epithelial cells exposed to SM, which take place within minutes to hours, are in accordance with the findings from the in vivo studies of forced weaning, demonstrating reversible changes in GLUT1 subcellular targeting within 3-5 hours (Nemeth et al., 2000). These findings are also supported by the previous observation, that as early as 15 minutes after exposure of mammary tissue fragments from lactating rabbits to prolactin, the cell morphology already changed with marked increase in the relative volume occupied by the Golgi region (Ollivier-Bousquet, 1978). This dynamic trafficking of GLUT1 under the influence of lactogenic hormones, with a reversible intracellular targeting of GLUT1 to the site of lactose synthesis in the Golgi within a relatively short time, offers another level of regulation of lactose synthesis.

The next step was to define which of the hormones in SM is responsible for GLUT1 intracellular targeting. Exposure of CIT₃ cells kept in GM to secretion medium containing prolactin without hydrocortisone caused the same changes in GLUT1 subcellular targeting. The same response was reproduced with prolactin concentrations as low as 3 ng/ml (compared to the 3 μ g/ml of prolactin usually used in SM) (Fig. 6, also available as a movie). The serum concentration range of prolactin in lactating mothers is 20-300 ng/ml (Rebar, 1999). We were not able to demonstrate dose-response effects with the different prolactin concentrations. GLUT1-EGFP intracellular signal translocation took place at approximately the same time (100-120 minutes) with 3 ng/ml prolactin, as it did with 3 μ g/ml of prolactin in the full SM. Possibly, further study could demonstrate dose-dependent effect of prolactin, expressed as different levels of the intracellular green

fluorescent signal of GLUT1 chimeras. This requires a more quantitative recording of the signal, which was beyond the scope of this current study. Secretion medium containing hydrocortisone without any prolactin caused no change in GLUT1 subcellular distribution, thus excluding its hormonal effect as the other possible cause for GLUT1 intracellular targeting in SM (Fig. 7, also available as a movie).

The suggestion that GLUT1 does not solely act at the plasma membrane, but may function in an intracellular organelle as well, conceptually complements the well-known insulin-regulated targeting of GLUT4, and to a lesser extent of GLUT1, to their site of action, the plasma membrane, in fat and muscle cells. The existence of a prolactin-induced, cell type-specific, developmental stage-specific sorting machinery for GLUT1 in mammary epithelial cells may also support glucose transport as a rate limiting step for lactose synthesis in the Golgi during lactation. The ability of the system to respond quickly to hormonal changes by altering the transport, and thus the availability of free glucose for lactose synthesis in the Golgi, is also complementary to this well-known insulin-regulated targeting of GLUT4 to the plasma membrane in fat and muscle cells, where GLUT4 is available for glucose uptake into the cell within minutes.

Sharing the same mechanisms of dynamic regulation by subcellular targeting raises the question, whether GLUT1 intracellular trafficking in the mammary epithelial cells may involve some of the endocytic and exocytic pathways involved in GLUT4 targeting in fat and muscle cells. Thus, in an effort to further define the intracellular trafficking mechanisms involved in GLUT1 targeting and recycling in mammary epithelial cells, agents which affect GLUT1 and GLUT4 targeting in muscle and fat cells were tested in our system.

Bafilomycin A1 ($C_{35}H_{58}O_9$) is an antibiotic that originates from *Streptomyces griseus*. It is a specific inhibitor of vacuolar proton pump type H^+ -ATPase (V-ATPase) in animal cells (Bowman et al., 1988). Thus, bafilomycin A1 causes arrest of endosomal acidification. It also inhibits the acidification of lysosomes and thus inhibits the degradation of proteins in lysosomes (Yoshimori et al., 1991). Bafilomycin A1 not only slows bulk membrane flow, but it causes additional inhibition of receptor recycling in a manner that is dependent on a peptide internalization motif on the cytoplasmic domain (Presley et al., 1997b). It was demonstrated that the removal of transferrin from sorting endosomes and accumulation in the peri-centriolar endocytic recycling compartment occurs in bafilomycin A1 treated cells, but the rate constant for transferrin receptors exit from the recycling endosomes was retarded. In fat and muscle cells, GLUT4, and to a lesser extent GLUT1 is constitutively sequestered in the endosomal tubulovesicular system, and moves to the cell surface in response to insulin. In muscle cells arrest of endosomal acidification by bafilomycin A1 results in rapid dose-dependent translocation of GLUT4 from the cell interior to the plasma membrane surface, mimicking insulin effect. This insulin-like effect of bafilomycin A1 causes redistribution of GLUT1 and Rab4, a regulatory component of the secretory and endocytic system, as well (Chinni and Shisheva, 1999). The mechanism by which arrest of endosomal acidification by bafilomycin A1 causes translocation of GLUT4 and GLUT1 is distal to the insulin receptor and phosphatidylinositol 3-kinase activation. It suggests an important role for endosomal pH in membrane dynamics and in the hormonally regulated intracellular sorting machinery of glucose transporters. Some of the studies done with bafilomycin A1 (Chinni and Shisheva, 1999; Yoshimori et al., 1991) also used acridine orange, which is

an acidotropic dye. This is readily taken by living cells and accumulates in intracellular acidified compartments (endosomes / lysosomes), giving rise to orange fluorescent signal, which disappears when these structures are alkalinized (Harada et al., 1997). Acridine orange was useless for our studies of time-lapse imaging due to a quick photosensitive changes in its pattern. Upon exposure to light, especially in the range used to detect green and red fluorescent signal, there is photo-oxidative disruption of the endosomal / lysosomal membranes (Brunk et al., 1997), which causes shift of the orange signal of acridine orange from these compartments into the nucleus, where it emits green fluorescent signal, due to photoenhancement phenomenon (De and Bhanja, 1976), obscuring the GLUT1-EGFP green intracellular signal. In CIT₃ kept in SM bafilomycin A1 caused central coalescence of GFP-GLUT1 and the loss of peripheral vesicles (Fig. 8-9, also available as movies). The effect was not reversible upon withdrawal of bafilomycin A1. We conclude that in living mouse mammary epithelial cells, prolactin causes intracellular targeting of GLUT1 by altering rates of GLUT1 exocytosis and endocytosis. Arrest of endosomal acidification by bafilomycin A1 disrupts this recycling process via endosomal compartments. The importance of endosomal pH to the prolactin-induced hormonally regulated sorting of GLUT1 in mammary epithelial cells, thus shares characteristics with GLUT4 and GLUT1 insulin-dependent intracellular redistribution in adipocytes. Further work can be done to delineate the membrane organization of GLUT1 recycling pathways in mammary epithelial cells, and define endosomal subpopulations involved in this process. Co-localization studies using Rab proteins (e.g. Rab4, Rab5 and Rab11) involved in the regulation of the transport machinery through distinct domains on the endosomes can be of value (Sonnichsen et al., 2000).

Wortmannin ($C_{23}H_{24}O_8$) is an antifungal antibiotic that originates from *Penicillium fumiculosum*. It is a highly specific inhibitor of phosphatidylinositol 3-kinase (PI3K), which is highly cell permeable. PI3K is necessary for insulin-stimulated glucose transport in myoblasts and adipocytes. In the presence of wortmannin GLUT1 and GLUT4 insulin-stimulated translocation to plasma membrane was inhibited (Clarke et al., 1994; Evans et al., 1995; Hausdorff et al., 1999; Kaliman et al., 1995; Wang et al., 1999). Interestingly, wortmannin also blocks a constitutive basal GLUT1 trafficking pathway in muscle cells, that involves PI3K but is independent of insulin. The result is that GLUT1 is sequestered in a perinuclear compartment (Kaliman et al., 1995; McDowell et al., 1997). This seems to be a more ubiquitous pathway used by other cell types for basal glucose uptake by GLUT1, as shown in fibroblasts (Jess et al., 1996; Young et al., 1995). GLUT1 protein appears to recycle between an intracellular site and the plasma membrane, as does the well-studied transferrin receptor protein. PI3K seems to have an important functional role in this recycling, regulating membrane protein traffic. By inhibition of PI3K, wortmannin causes selective blockade of this protein recycling with accumulation of glucose transporters in intracellular location in fibroblasts (Jess et al., 1996). In CIT₃ mouse mammary epithelial cells, wortmannin causes internalization of GFP-GLUT1 plasma membrane signal in GM (Fig. 10, also available as a movie). We were not able to demonstrate reversibility of the effect upon withdrawal of the wortmannin. No visible effect was seen in SM. The effects of wortmannin on GLUT1 in living mouse mammary epithelial cells are thus independent of prolactin, and seem to be related to blockade of the constitutive basal GLUT1 trafficking pathway, described above. These findings also further support the role of recycling of GLUT1 by exocytosis

and endocytosis. Study of the effects of wortmannin on the endosomal system and GLUT4 recycling compartments in adipocytes (Malide and Cushman, 1997) supports the role of endosomal derived vacuoles and endosomal recycling compartments, sensitive to wortmannin, for this process. The effect of wortmannin on decreasing prolactin-induced lactose synthesis (Hang and Rillema, 1998) was related to inhibition of PI3K upstream in the prolactin signaling pathway for p70 ribosomal S6 kinase ($P70^{S6K}$), and was seen in the time range of 12-19 hours. The lack of effect of wortmannin on GLUT1 targeting in SM seen by us, implies that PI3K is not involved in the pathways controlling the relatively quick prolactin-induced GLUT1 change in intracellular targeting, which we demonstrated. In fibroblasts PI3K, but not $P70^{S6K}$, was shown to be involved in GLUT1 protein membrane recycling (Jess et al., 1996), further supporting our findings of wortmannin effects in GM, but not in SM. The effect of wortmannin in GM is related to PI3K role only in basal membrane protein trafficking, which is not hormonally regulated by prolactin.

Staurosporine ($C_{28}H_{26} N_4O_3$) is an antibiotic that originates from Streptomyces species. It is a potent inhibitor of protein kinase C (PKC). In fat and muscle cells insulin increases glucose uptake via a PKC-dependent pathway. Staurosporine inhibits GLUT1 and GLUT4 trafficking to plasma membrane in adipocytes (Nishimura and Simpson, 1994). PKC is involved in insulin signaling in other cell types as well, i.e. fibroblasts (Cooper et al., 1999), resulting in GLUT1 recruitment to plasma membrane. But PKC is also involved in translocation of GLUT3 to plasma membrane in activated platelets in response to thrombin (Sorbara et al., 1997), suggesting a common PKC activation-mediated signalling pathway for recruitment of glucose transporters to the cell surface to

increase glucose uptake (McCoy et al., 1997). In CIT₃ mouse mammary epithelial cells in GM staurosporine causes internalization of GFP-GLUT1 plasma membrane signal (Fig. 11, also available as a movie). In SM, it causes central coalescence of GFP-GLUT1 and loss of peripheral vesicles (Fig. 12, also available as a movie). These findings support the role of PKC in the sorting machinery controlling GLUT1 recycling in mammary epithelial cells, as in many other cells, as described above. Based on lack of other evidence from the literature, and the fact that staurosporine had similar effects in GM and SM, we cannot establish tissue-specific or prolactin-induced role for PKC in the control of GLUT1 intracellular trafficking and targeting.

Future studies will aim at defining the underlying mechanism that allows this relatively rapid changes in GLUT1 subcellular targeting in response to prolactin. Possibly, this is related to phosphorylation or dephosphorylation reaction in response to prolactin.

Another subject that merits further study is the role of subpopulations of endosomes and endosomal recycling compartments in GLUT1 basal recycling (Malide and Cushman, 1997), and moreover in its intracellular trafficking and targeting in secretion medium, as supported by the marked effects seen with the arrest of endosomal acidification by bafilomycin A1. Co-localization studies using Rab proteins (e.g. Rab4, Rab5 and Rab11) involved in the regulation of the transport machinery through distinct domains on the endosomes have shown that endosomes are organized into compartments within the same continuous membrane, which cooperatively generate a recycling continuum. (Sonnichsen et al., 2000).

In summary, our results demonstrate a basal constitutive GLUT1 membrane-recycling pathway between an intracellular pool and the cell surface in mouse mammary epithelial cells, which targets most of the GLUT1 to the plasma membrane in GM. This basal recycling and sorting process is common to other cell types (Jess et al., 1996), and is regulated by PI3K and PKC, thus blocked by wortmannin and staurosporine. As in other cell types it is responsible for maintaining basal glucose uptake. But, in mammary epithelial cells there is also another hormonally-regulated cell type-specific, developmental stage-specific sorting machinery for GLUT1 intracellular targeting in lactation. This process is induced by prolactin, and is highly sensitive to low concentrations of prolactin. It provides the cell a quick mechanism, by which it can supply free glucose intracellularly, where it serves as substrate for lactose synthesis in the Golgi. This machinery offers another level of regulation of lactose synthesis by altering GLUT1 targeting within minutes to hours, as was demonstrated also *in vivo* (Nemeth et al., 2000). This step may not require new protein synthesis, or increase in the total amount of GLUT1 or enzymes involved in lactose synthesis, which take longer. It may also support glucose transport as a rate-limiting step for lactose synthesis in the Golgi during lactation.

FIGURE LEGENDS

Figure 1 - CIT₃ cells expressing EGFP fusion to the N- and C-termini of GLUT1:

High power images. The high-resolution images with the green fluorescent signal of EGFP allow better demonstration of the change in GLUT1 targeting from plasma membrane to intracellular perinuclear punctate pattern. This occurs when differentiation is stimulated by changing medium from growth media (GM) to secretion media (SM) for four days. A - Plasma membrane targeting of EGFP-GLUT1 in GM. B – Intracellular pattern of EGFP-GLUT1 signal in differentiated cells in SM. C - Plasma membrane targeting of GLUT1-EGFP in GM. D – Intracellular targeting of GLUT1-EGFP with probable Golgi distribution pattern in cells exposed to SM. All images captured with $\frac{1}{4}$ sec exposure time and are at 40x magnification, except for Fig A, which was optimized at $\frac{1}{2}$ sec exposure time and higher power (60x).

Figure 2: GLUT1 chimeras to GFP co-localize with native GLUT1:

Immunocytochemistry studies with antibodies against the C-terminus of native GLUT1 showed that the fluorescent signal of both GLUT1 chimeras to GFP co-localized with native GLUT1 in SM.

Figure 3: GLUT1 intracellular trafficking in SM is dynamic:

Static images of cells at different levels of differentiation in SM demonstrate that dynamic transport systems are involved in the trafficking of GFP-GLUT1 fusion proteins. 4 panells. Upper left in GM, the rest in SM at different level of differentiation. The upper left figure of each panel is GLUT1-EGFP signal. The upper right figure is staining of the same cells with BODIPY-TR-ceramide (Molecular Probes, Inc. Eugene, OR), which is a dye that marks the trans-Golgi in red. Living CIT₃ cells were pre-incubated with 5

nmol/ml of BODIPY-TR-ceramide at 4°C for 30 minutes. Lower left figure is superimposed image of the upper figures. Lower right figure is phase contrast image of the cells to show the different levels of differentiation.

Figure 4: GLUT1 intracellular trafficking in SM is dynamic:

Living CIT₃ cells transfected with GLUT1-EGFP and kept in SM are followed by time-lapse imaging, where dynamic trafficking of GLUT1 fusion proteins can be seen (Fig. 4, also available as movie).

Figure 5: Lactogenic hormones in secretion medium induces GLUT1 intracellular targeting in living mammary cells:

Living CIT3 cells, transfected with GLUT1-EGFP and kept in GM, are exposed to SM. Dynamic trafficking of GLUT1 fusion proteins intracellularly is demonstrated, starting after approximately 50-60 minutes, with maximal intracellular targeting within 90-110 minutes. When the cells are returned to GM, most of the changes are reversible within 1-2 hours, although not fully, with redistribution of the fluorescent GLUT1 chimera mostly in the plasma membrane (Fig. 5, also available as movie).

Figure 6: Prolactin induces GLUT1 intracellular targeting in living mammary cells:

Exposure of CIT₃ cells kept in GM to secretion medium containing prolactin without hydrocortisone causes intracellular targeting of GLUT1. Dynamic trafficking of GLUT1 fusion proteins intracellularly is demonstrated, starting after approximately 50-60 minutes, with maximal intracellular targeting within 90-110 minutes. When the cells are returned to GM, most of the changes are reversible within 1-2 hours, although not fully. The response was reproduced with prolactin concentrations of 3 ng/ml (compared to the 3 µg/ml of prolactin usually used in SM, see Fig. 5) (Fig. 6, also available as a movie).

Figure 7: Hydrocortisone does not induce GLUT1 intracellular targeting in living mammary epithelial cells:

Secretion medium containing hydrocortisone (3 μ g/ml as in full SM) without any prolactin caused no change in GLUT1 subcellular distribution (Fig. 7, also available as a movie).

Figure 8: Bafilomycin A1 causes central coalescence of GLUT1-EGFP with loss of peripheral vesicles in SM:

CIT₃ transfected with GLUT1-EGFP and kept in SM for 96 hours show central coalescence of GLUT1-EGFP green signal with the loss of peripheral vesicles after treatment with 800 nM of bafilomycin A1. The effect was seen after approximately 1 hour and was not reversible upon withdrawal of bafilomycin A1 (Fig. 8, also available as movies).

Figure 9: Bafilomycin A1 causes central coalescence of GLUT1-EGFP with loss of peripheral vesicles in SM:

CIT₃ transfected with GLUT1-EGFP and kept in SM for 96 hours show central coalescence of GLUT1-EGFP green signal with the loss of peripheral vesicles after treatment with 800 nM of bafilomycin A1. The effect was seen after approximately 1 hour and was not reversible upon withdrawal of bafilomycin A1 (Fig. 9, also available as movies).

Figure 10: Wortmannin causes internalization of GLUT1 signal in GM:

In GM 1 μ M of wortmannin causes internalization of GLUT1-EGFP plasma membrane signal. The effect was seen after approximately 1-2 hours. No reversibility of the effect

could be demonstrated upon withdrawal of the wortmannin (Fig. 10, also available as a movie).

Figure 11: Staurosporine causes internalization of GLUT1-EGFP in GM:

In GM staurosporine causes internalization of GLUT1-EGFP plasma membrane signal. This set of time lapse images also suggests a possible intracellular and intercellular network which constitutes the pathway for internalization of the GLUT1-EGFP vesicles (Fig. 11, also available as a movie).

Figure 12: Staurosporine causes central coalescence of GLUT1-EGFP vesicles in SM:

In CIT₃ cells exposed to SM for 96 hours, staurosporine causes central coalescence of GLUT1-EGFP signal and loss of peripheral vesicles signal (Fig. 12, also available as a movie).

REFERENCES

Bowman, E.J., A. Siebers, and K. Altendorf. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc Natl Acad Sci U S A.* 85:7972-6.

Brunk, U.T., H. Dalen, K. Roberg, and H.B. Hellquist. 1997. Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. *Free Radic Biol Med.* 23:616-26.

Burnol, A.F., A. Leturque, M. Loizeau, C. Postic, and J. Girard. 1990. Glucose transporter expression in rat mammary gland. *Biochem J.* 270:277-9.

Camps, M., S. Vilaro, X. Testar, M. Palacin, and A. Zorzano. 1994. High and polarized expression of GLUT1 glucose transporters in epithelial cells from mammary gland: acute down-regulation of GLUT1 carriers by weaning. *Endocrinology.* 134:924-34.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science.* 263:802-5.

Chinmi, S.R., and A. Shisheva. 1999. Arrest of endosome acidification by bafilomycin A1 mimics insulin action on GLUT4 translocation in 3T3-L1 adipocytes. *Biochem J.* 339:599-606.

Clarke, J.F., P.W. Young, K. Yonezawa, M. Kasuga, and G.D. Holman. 1994. Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. *Biochem J.* 300:631-5.

Cooper, D.R., J.E. Watson, N. Patel, P. Illingworth, M. Acevedo-Duncan, J. Goodnight, C.E. Chalfant, and H. Mischak. 1999. Ectopic expression of protein kinase

CbetaII, -delta, and -epsilon, but not -betaI or -zeta, provide for insulin stimulation of glucose uptake in NIH-3T3 cells. *Arch Biochem Biophys.* 372:69-79.

Cormack, B.P., R.H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene.* 173:33-8.

De, D.N., and P. Bhanja. 1976. A phenomenon of photoenhancement of acridine orange-induced fluorescence. *J Histochem Cytochem.* 24:674-8.

Dobson, S.P., C. Livingstone, G.W. Gould, and J.M. Tavare. 1996. Dynamics of insulin-stimulated translocation of GLUT4 in single living cells visualised using green fluorescent protein. *FEBS Lett.* 393:179-84.

Drmota, T., G.W. Gould, and G. Milligan. 1998. Real time visualization of agonist-mediated redistribution and internalization of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor. *J Biol Chem.* 273:24000-8.

Evans, J.L., C.M. Honer, B.E. Womelsdorf, E.L. Kaplan, and P.A. Bell. 1995. The effects of wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase, on insulin-stimulated glucose transport, GLUT4 translocation, antilipolysis, and DNA synthesis. *Cell Signal.* 7:365-76.

Garcia-Mata, R., Z. Bebok, E.J. Sorscher, and E.S. Sztul. 1999. Characterization and dynamics of aggresome formation by a cytosolic GFP- chimera. *J Cell Biol.* 146:1239-54.

Gould, G.W., V. Derechin, D.E. James, K. Tordjman, S. Ahern, E.M. Gibbs, G.E. Lienhard, and M. Mueckler. 1989. Insulin-stimulated translocation of the

HepG2/erythrocyte-type glucose transporter expressed in 3T3-L1 adipocytes. *J Biol Chem.* 264:2180-4.

Haas, J., E.C. Park, and B. Seed. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol.* 6:315-24.

Hang, J., and J.A. Rillem. 1998. Possible involvement of P13K in prolactin-stimulated milk product formation and iodide transport in mouse mammary explants. *Proc Soc Exp Biol Med.* 219:154-9.

Harada, M., S. Shakado, S. Sakisaka, S. Tamaki, M. Ohishi, K. Sasatomi, H. Koga, M. Sata, and K. Tanikawa. 1997. Bafilomycin A1, a specific inhibitor of V-type H⁺-ATPases, inhibits the acidification of endocytic structures and inhibits horseradish peroxidase uptake in isolated rat sinusoidal endothelial cells. *Liver.* 17:244-50.

Hausdorff, S.F., D.C. Fingar, K. Morioka, L.A. Garza, E.L. Whiteman, S.A. Summers, and M.J. Birnbaum. 1999. Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. Dissociation of insulin-stimulated glucose uptake and GLUT4 translocation. *J Biol Chem.* 274:24677-84.

Jess, T.J., C.M. Belham, F.J. Thomson, P.H. Scott, R.J. Plevin, and G.W. Gould. 1996. Phosphatidylinositol 3'-kinase, but not p70 ribosomal S6 kinase, is involved in membrane protein recycling: wortmannin inhibits glucose transport and downregulates cell-surface transferrin receptor numbers independently of any effect on fluid-phase endocytosis in fibroblasts. *Cell Signal.* 8:297-304.

Kaether, C., T. Salm, M. Glombik, W. Almers, and H.H. Gerdes. 1997. Targeting of green fluorescent protein to neuroendocrine secretory granules: a new tool for real time studies of regulated protein secretion. *Eur J Cell Biol.* 74:133-42.

Kain, S.R., M. Adams, A. Kondepudi, T.T. Yang, W.W. Ward, and P. Kitts. 1995. Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques*. 19:650-5.

Kaliman, P., F. Vinals, X. Testar, M. Palacin, and A. Zorzano. 1995. Disruption of GLUT1 glucose carrier trafficking in L6E9 and Sol8 myoblasts by the phosphatidylinositol 3-kinase inhibitor wortmannin. *Biochem J*. 312:471-7.

Kida, E., W. Kaczmarski, A.A. Golabek, A. Kaczmarski, M. Michalewski, and K.E. Wisniewski. 1999. Analysis of intracellular distribution and trafficking of the CLN3 protein in fusion with the green fluorescent protein in vitro. *Mol Genet Metab*. 66:265-71.

Lippincott-Schwartz, J., N. Cole, and J. Presley. 1998. Unravelling Golgi membrane traffic with green fluorescent protein chimeras. *Trends Cell Biol*. 8:16-20.

Madon, R.J., S. Martin, A. Davies, H.A. Fawcett, D.J. Flint, and S.A. Baldwin. 1990. Identification and characterization of glucose transport proteins in plasma membrane- and Golgi vesicle-enriched fractions prepared from lactating rat mammary gland. *Biochem J*. 272:99-105.

Malide, D., and S.W. Cushman. 1997. Morphological effects of wortmannin on the endosomal system and GLUT4-containing compartments in rat adipose cells. *J Cell Sci*. 110:2795-806.

McCoy, K.D., N. Ahmed, A.S. Tan, and M.V. Berridge. 1997. The hemopoietic growth factor, interleukin-3, promotes glucose transport by increasing the specific activity and maintaining the affinity for glucose of plasma membrane glucose transporters. *J Biol Chem*. 272:17276-82.

McDowell, H.E., T. Walker, E. Hajduch, G. Christie, I.H. Batty, C.P. Downes, and H.S. Hundal. 1997. Inositol phospholipid 3-kinase is activated by cellular stress but is not required for the stress-induced activation of glucose transport in L6 rat skeletal muscle cells. *Eur J Biochem*. 247:306-13.

Mueckler, M., C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard, and H.F. Lodish. 1985. Sequence and structure of a human glucose transporter. *Science*. 229:941-5.

Nemeth, B.A., S.W. Tsang, R.S. Geske, and P.M. Haney. 2000. Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland. *Pediatr Res*. 47:444-50.

Nishimura, H., and I.A. Simpson. 1994. Staurosporine inhibits phorbol 12-myristate 13-acetate- and insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters in rat adipose cells. *Biochem J*. 302:271-7.

Ollivier-Bousquet, M. 1978. Early effects of prolactin on lactating rabbit mammary gland. Ultrastructural changes and stimulation of casein secretion. *Cell Tissue Res*. 187:25-43.

Patterson, G.H., S.M. Knobel, W.D. Sharif, S.R. Kain, and D.W. Piston. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys J*. 73:2782-90.

Presley, J.F., N.B. Cole, T.A. Schroer, K. Hirschberg, K.J. Zaal, and J. Lippincott-Schwartz. 1997a. ER-to-Golgi transport visualized in living cells [see comments]. *Nature*. 389:81-5.

Presley, J.F., S. Mayor, T.E. McGraw, K.W. Dunn, and F.R. Maxfield. 1997b. Bafilomycin A1 treatment retards transferrin receptor recycling more than bulk membrane recycling. *J Biol Chem.* 272:13929-36.

Rebar, R.W. 1999. The breast and the physiology of lactation. In *Maternal-Fetal Medicine*. R.K. Creasy and R. Resnik, editors. W.B. Saunders, Philadelphia. 106-121.

Shennan, D.B. 1998. Mammary gland membrane transport systems. *J. Mammary Biol. and Neoplasia.* 3:247-258.

Sonnichsen, B., S. De Renzis, E. Nielsen, J. Rietdorf, and M. Zerial. 2000. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J Cell Biol.* 149:901-14.

Sorbara, L.R., T.M. Davies-Hill, E.M. Koehler-Stec, S.J. Vannucci, M.K. Horne, and I.A. Simpson. 1997. Thrombin-induced translocation of GLUT3 glucose transporters in human platelets. *Biochem J.* 328:511-6.

Strous, G.J. 1986. Golgi and secreted galactosyltransferase. *CRC Crit Rev Biochem.* 21:119-51.

Takata, K., K. Fujikura, M. Suzuki, T. Suzuki, and H. Hirano. 1997. GLUT1 glucose transporter in the lactating mammary gland in the rat. *Acta Histochem Cytochem.* 30:623-8.

Toddywalla, V.S., F.W. Kari, and M.C. Neville. 1997. Active transport of nitrofurantoin across a mouse mammary epithelial monolayer. *J Pharmacol Exp Ther.* 280:669-76.

Tsien, R.Y. 1998. The green fluorescent protein. *Annu Rev Biochem.* 67:509-44.

Wang, L., H. Hayashi, and Y. Ebina. 1999. Transient effect of platelet-derived growth factor on GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem.* 274:19246-53.

Yang, T.T., P. Sinai, G. Green, P.A. Kitts, Y.T. Chen, L. Lybarger, R. Chervenak, G.H. Patterson, D.W. Piston, and S.R. Kain. 1998. Improved fluorescence and dual color detection with enhanced blue and green variants of the green fluorescent protein. *J Biol Chem.* 273:8212-6.

Yoshimori, T., A. Yamamoto, Y. Moriyama, M. Futai, and Y. Tashiro. 1991. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J Biol Chem.* 266:17707-12.

Young, A.T., J. Dahl, S.F. Hausdorff, P.H. Bauer, M.J. Birnbaum, and T.L. Benjamin. 1995. Phosphatidylinositol 3-kinase binding to polyoma virus middle tumor antigen mediates elevation of glucose transport by increasing translocation of the GLUT1 transporter [published erratum appears in Proc Natl Acad Sci U S A 1996 Jan 23;93(2):961]. *Proc Natl Acad Sci U S A.* 92:11613-7.

FIGURE 1



FIGURE 2

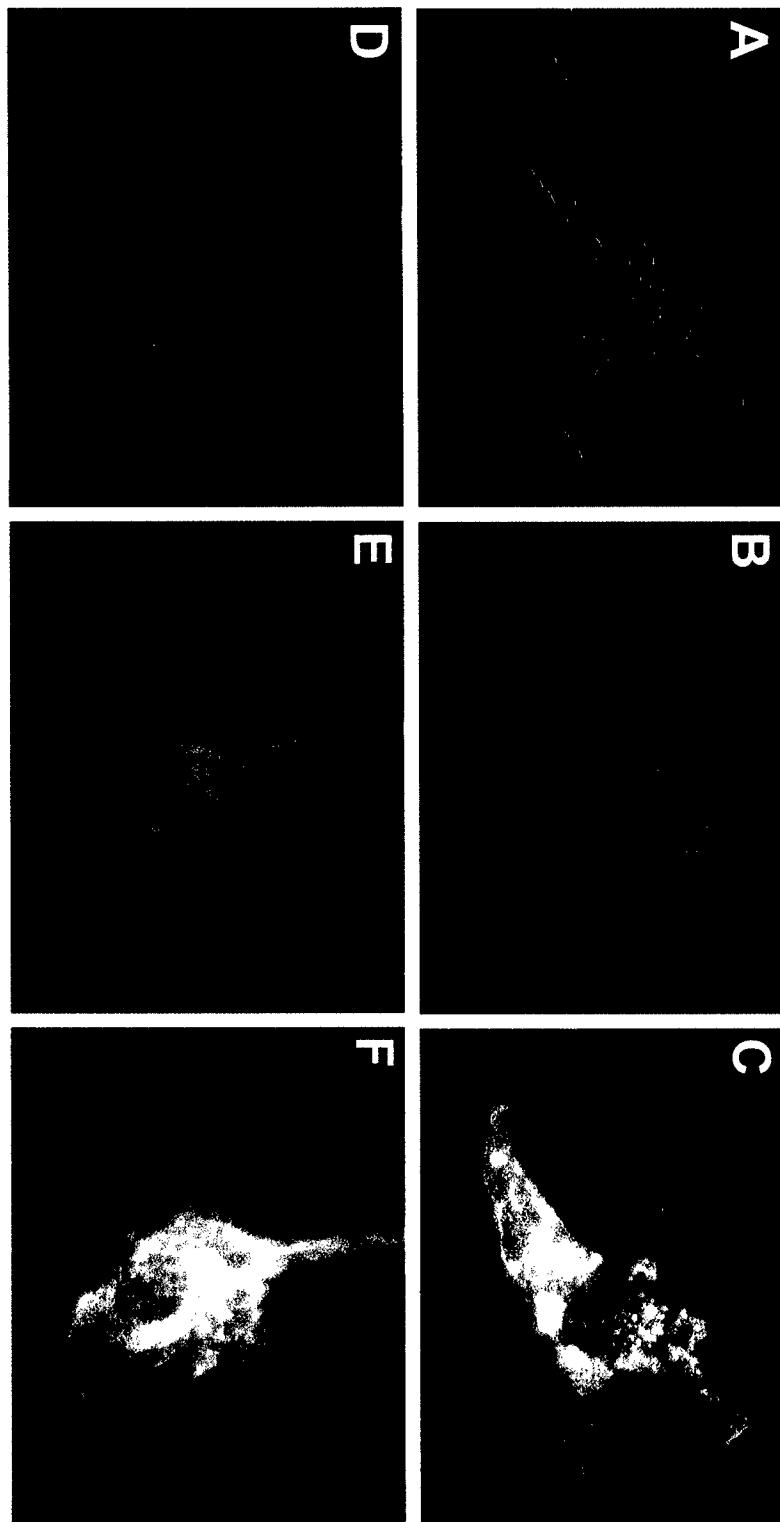


FIGURE 3

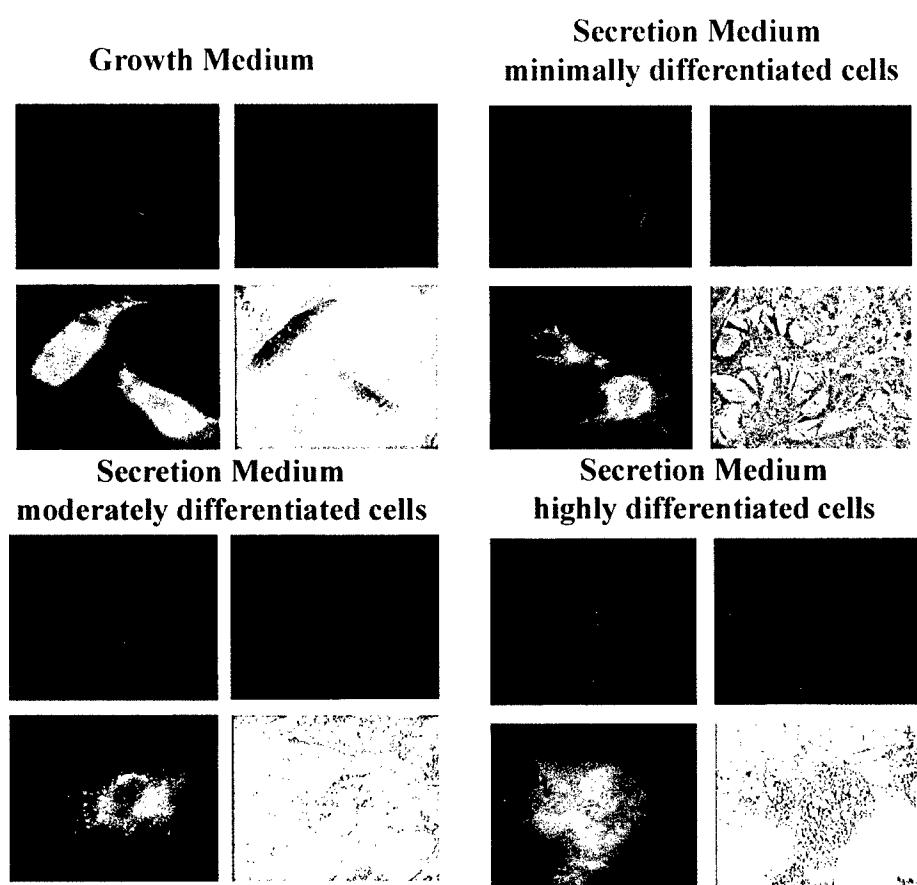


FIGURE 4

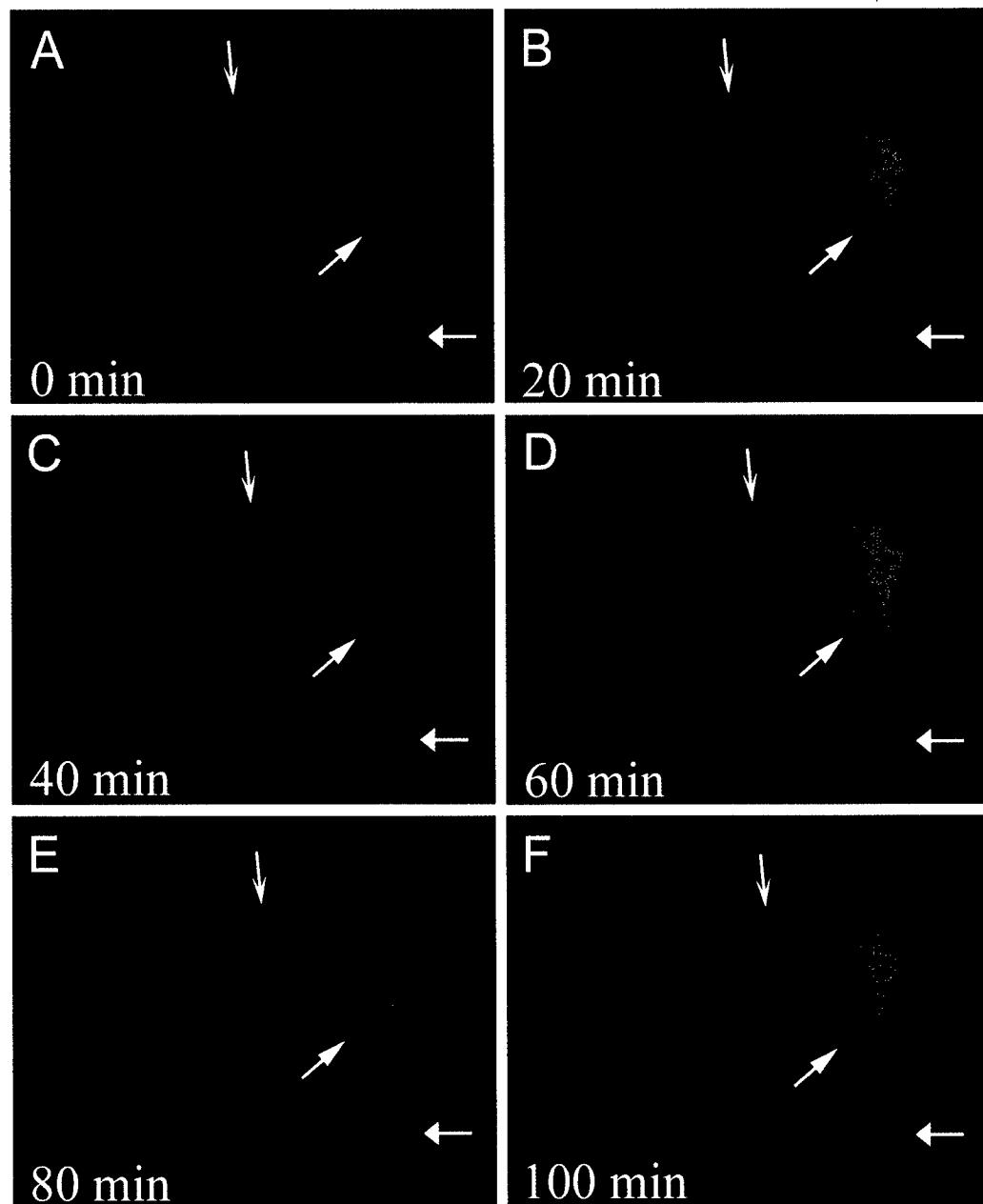


FIGURE 5

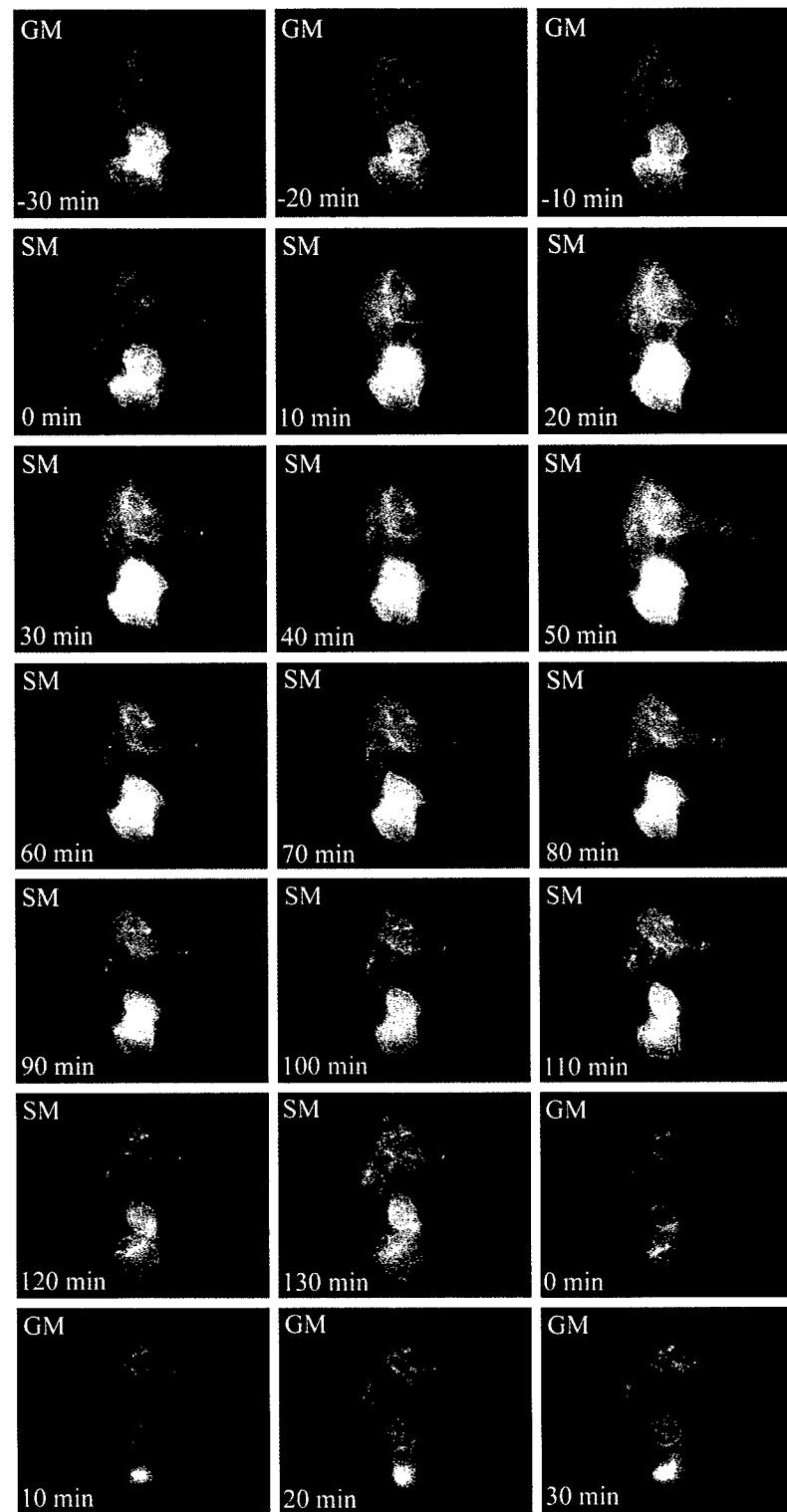


FIGURE 6

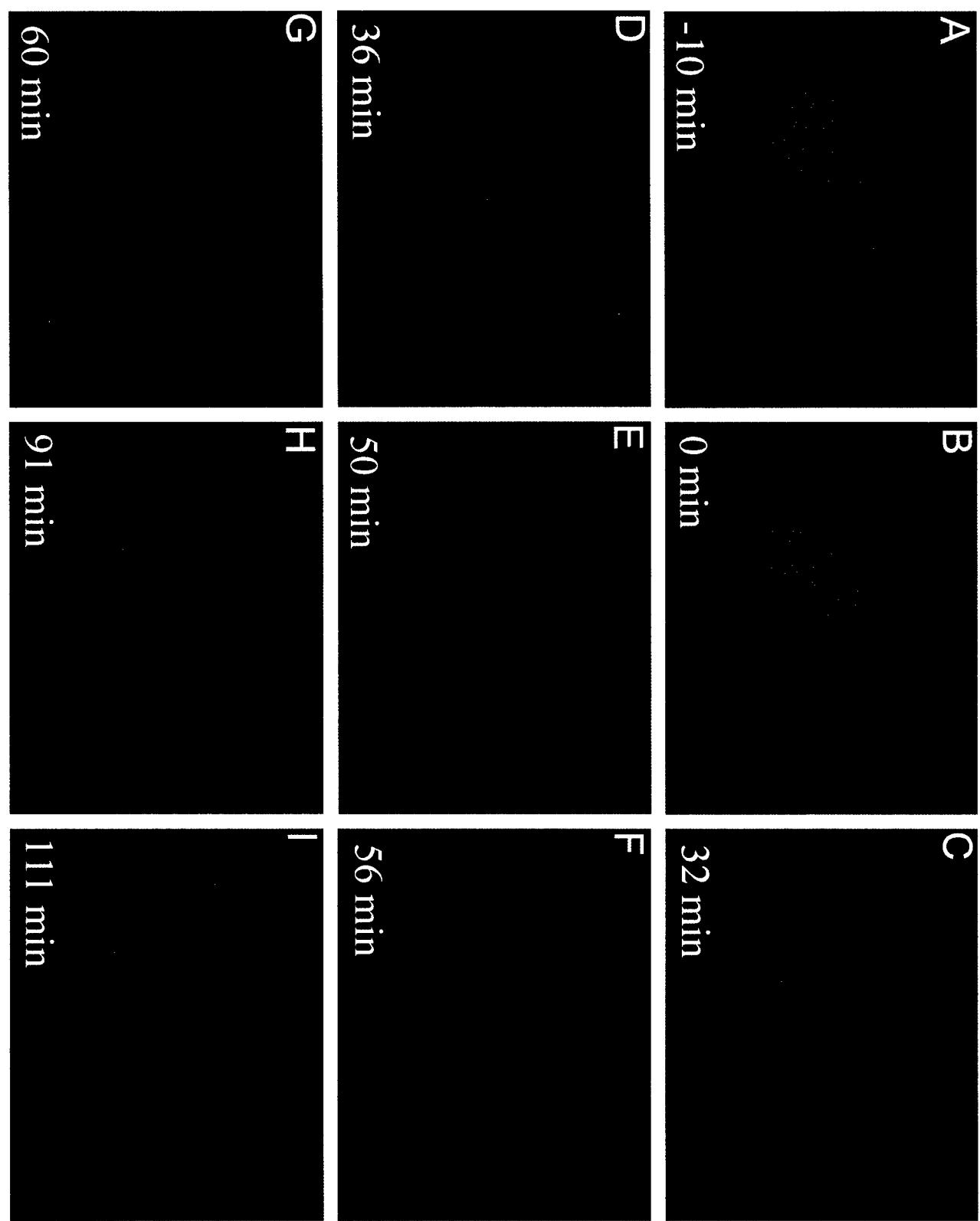


FIGURE 7

GM	SM	SM
-10 min	0 min	10 min
SM	SM	SM
20 min	30 min	40 min
SM	SM	SM
50 min	60 min	70 min
SM	SM	SM
80 min	90 min	100 min

FIGURE 8

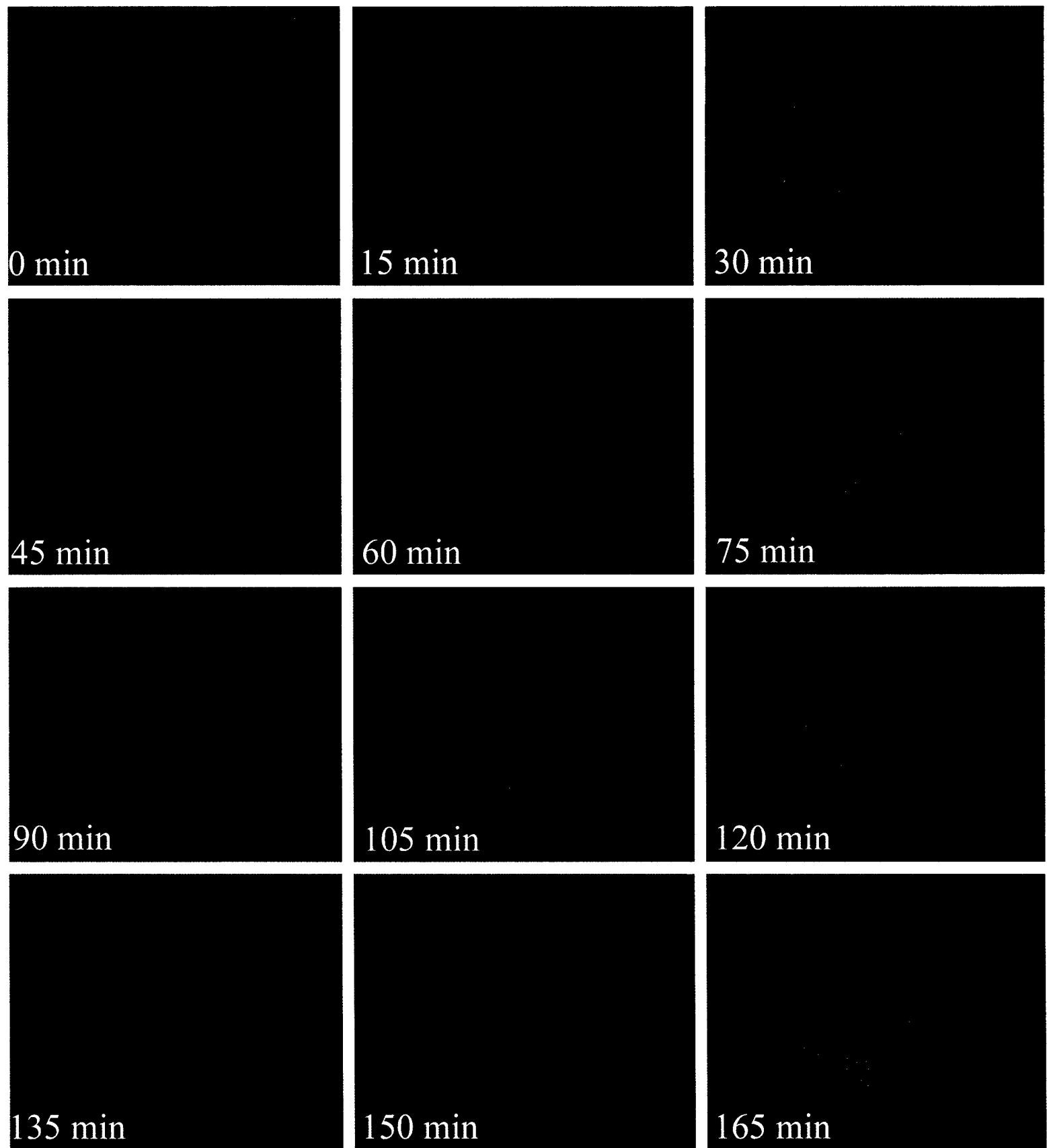


FIGURE 9

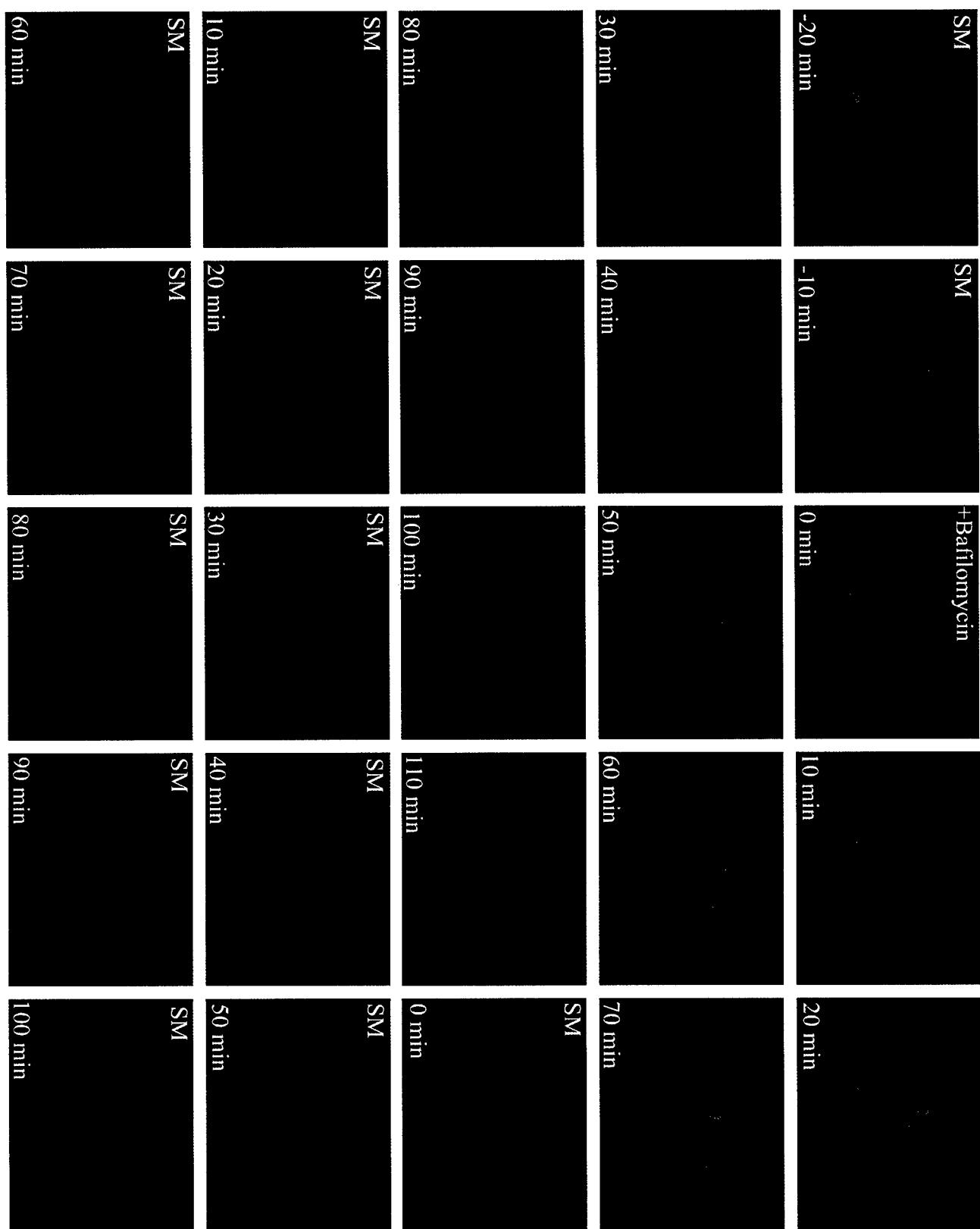


FIGURE 10

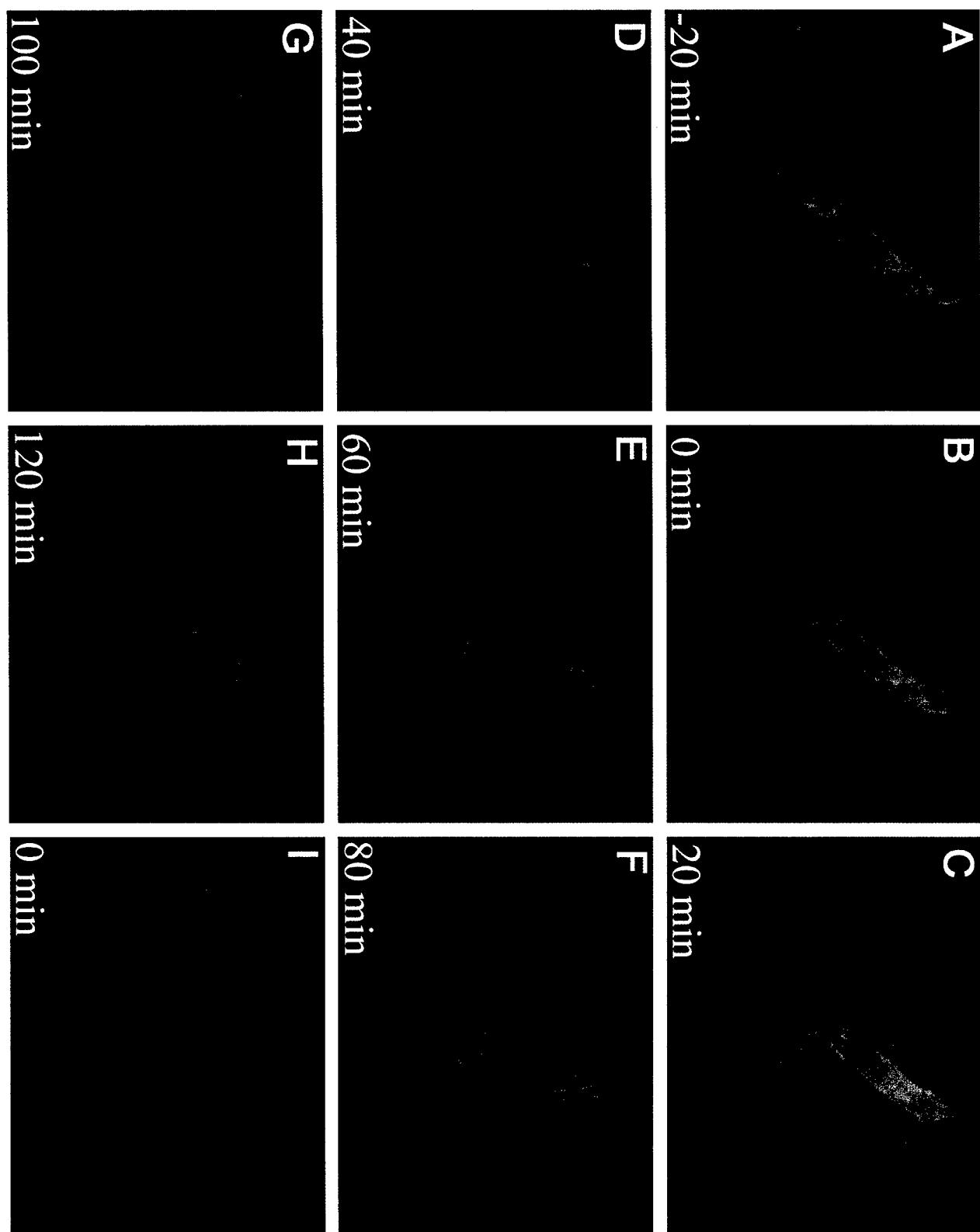


FIGURE 11

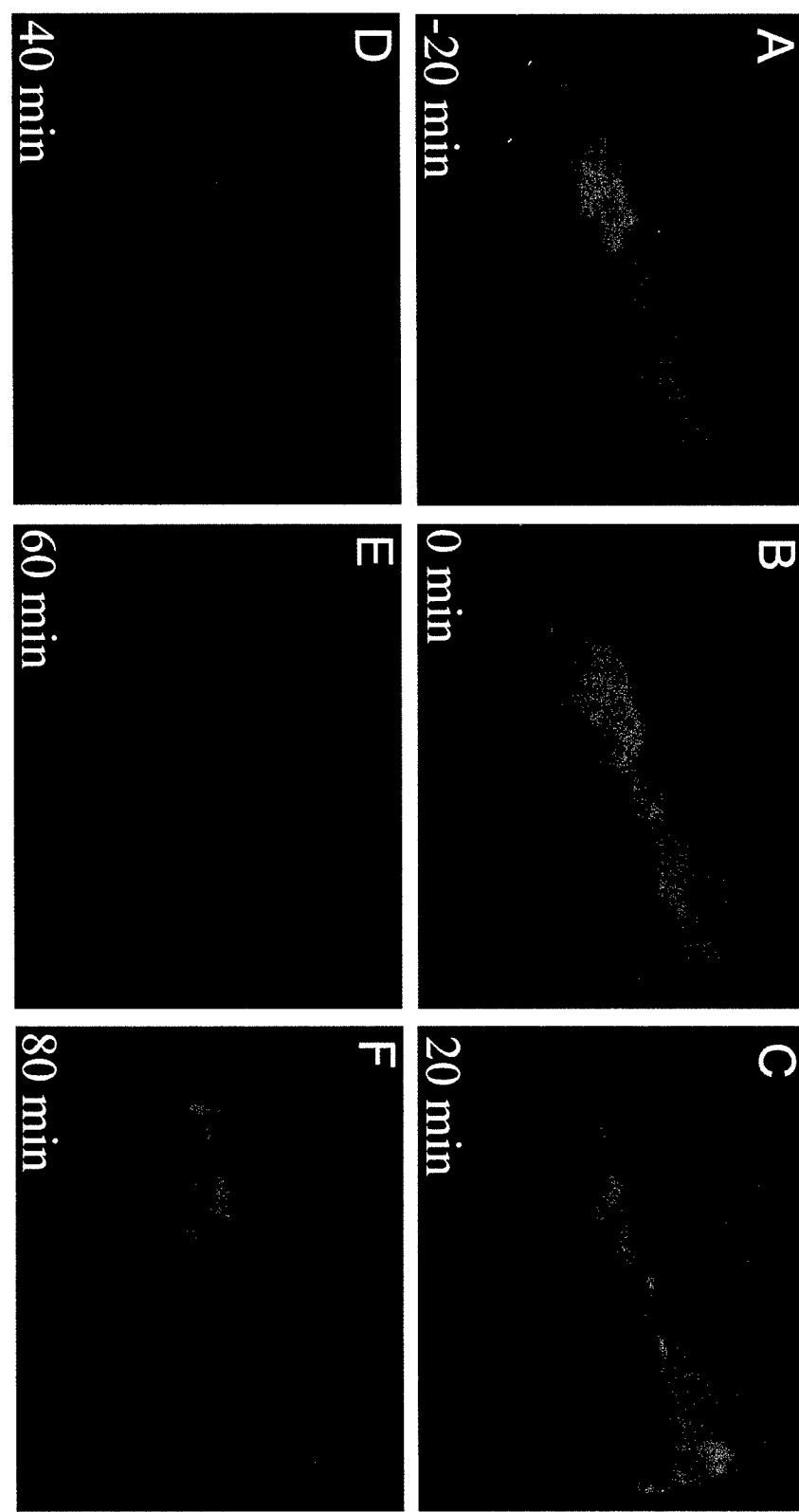
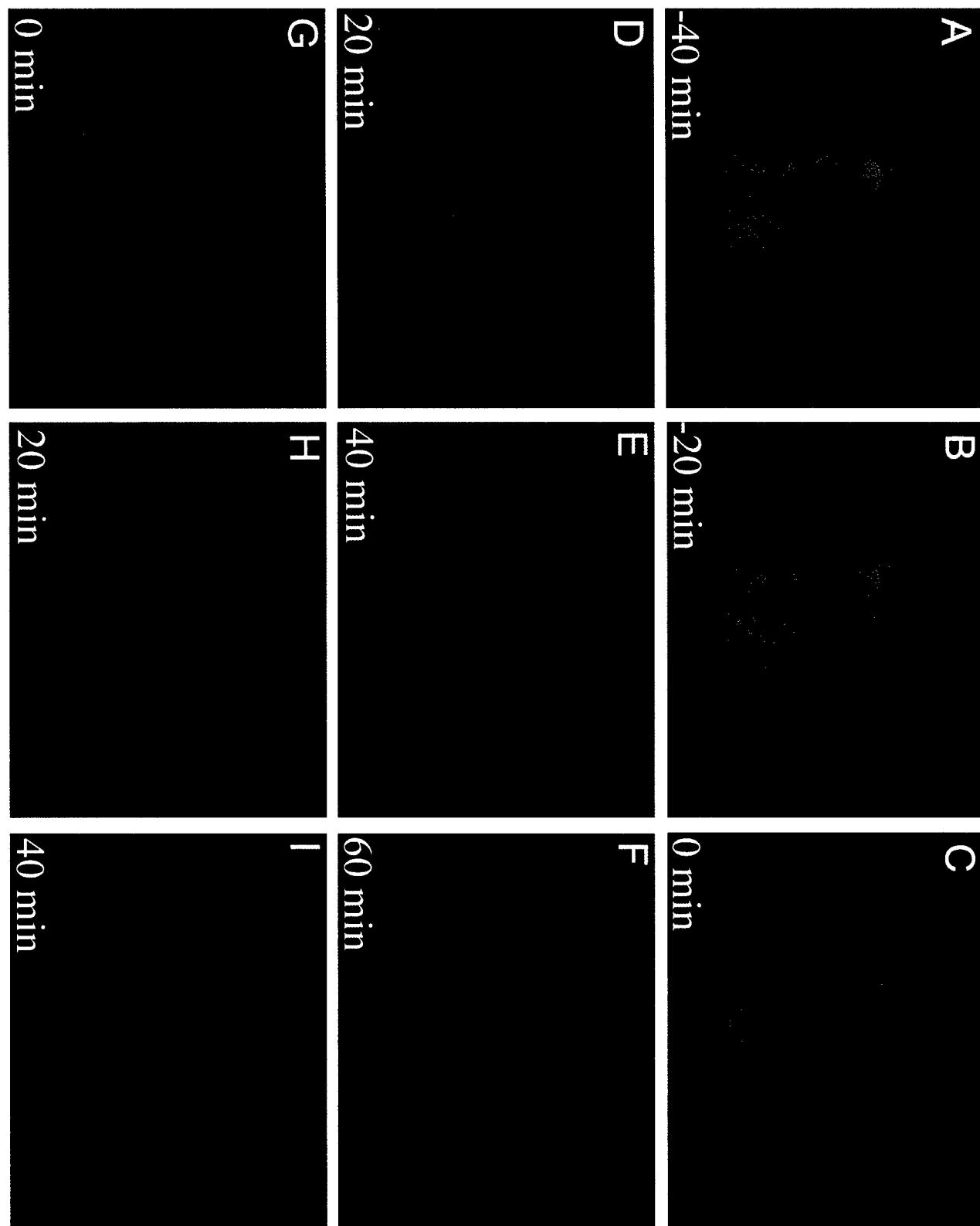


Figure 12





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.
2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

Phylis Rinehart
PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

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